

Plant Desiccation Tolerance

Editors

MATTHEW A. JENKS, PhD

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Preface

Over the past decade, our understanding of plant adaptation to extreme conditions, including the ability of plants and their parts to survive severe cellular desiccation, has grown considerably. While the general definition of desiccation simply refers to a reduction in water content, the scientific use of the term has come to define a more specific set of conditions wherein internal water stores have dropped to such a low level (often below 25% of full hydration) that cell turgidity and the integrity of internal membranes and proteins is severely compromised. Very few plants are capable of tolerating this kind of desiccation stress in their foliar tissues, and then recovering fully upon re-watering. Desiccation tolerant plants are generally referred to as resurrection plants, and include a diverse collection of plant species.

Most plants avoid harmful reductions in internal water supplies by using a variety of adaptive mechanisms such as increasing water uptake from the soil by increasing the size of their root systems, reducing transpirational water loss by closing stomata, reducing cuticular permeability, reducing leaf surface area, or using CAM metabolism. These mechanisms however do not provide protection to the extreme tissue and cellular desiccation that can occur as water becomes increasingly limited or unavailable. Desiccation tolerant plants possess unique physiological and biochemical mechanisms for surviving extreme dryness of their vegetative tissues. It is important to note however that desiccation tolerance is an adaptive trait found in most terrestrial plants as well, and not just resurrection plants. For instance, a common adaptation for dry-land survival is the ability to condition dormancy during periods when water is limiting, most often involving a highly desiccated (dry) seed stage. Reproductive structures like pollen and spores are also able to survive severe desiccation.

As aquifers are depleted to supply irrigation for expanding agricultural production worldwide, the potential for crop losses to drought becomes more acute. Even today, over-utilization of these dwindling water supplies is leading to an ever-increasing area where productive farming itself has ceased or is threatened. New and improved crops with reduced water requirements and increased drought tolerance could benefit agriculture globally by reducing the use of groundwater resources, and by expanding the productivity and sustainability of crops on existing and new lands. Recent scientific discoveries about desiccation tolerance in plants are not only revealing new insights into this

novel adaptation, but also shedding light on means by which these discoveries might be used to improve the drought tolerance of economically important crops. As such, this new understanding of desiccation tolerance determinants could lay the groundwork for using biotechnology to improve crops, and provide an important part of future water resource management decisions.

This book seeks to summarize the large body of current knowledge associated with cellular and organismal mechanisms of desiccation tolerance. Leading scientists in plant desiccation stress research worldwide provide a comprehensive treatise to the major factors controlling plant desiccation response and adaptation. The material presented in this book emphasizes fundamental genetic, physiological, biochemical, and ecological knowledge of plant desiccation tolerance mechanisms, knowledge that could one day lead to useful applications for the improvement of crop performance in dry-land environments.

We, the editors, would like to give a special thanks to the authors for their outstanding and timely work in producing such fine chapters. We would also like to thank Katie Vanvehoven for her clerical assistance, and Blackwell Publishing's Erica Judisch for her advice and encouragement during the development of this important book.

Matthew A. Jenks and Andrew J. Wood

Section 1

Vegetative Desiccation Tolerance

1 Plant Desiccation Tolerance: Diversity, Distribution, and Real-World Applications

Andrew J. Wood and Matthew A. Jenks

1.1 Introduction

Water is the key component of life, and organisms exhibit a suite of adaptations for surviving and thriving within a water-limited environment (Wood 2005). Terrestrial organisms are constantly losing water to the surrounding environment because they are in disequilibria with the atmosphere (i.e., the surrounding air is extremely “dry” relative to the organism). The vast majority of organisms cannot survive equilibrium with dry air and will die upon complete drying. However, a number of species *can* survive complete drying and “resurrect from the dead.”

An excellent example of a resurrection plant is the “common cushion moss” *Grimmia laevigata* (Brid.) Brid. Cushion mosses are found throughout the world in a variety of water-limiting environments—from arid and semiarid regions to dry areas within the landscape such as exposed rock formations (Keever 1957, Fernandez et al. 2006). The moss will naturally dry and equilibrate with the ambient air; photosynthesis and respiration are undetectable within the moss, and baking the sample will not “drive off” any more free water. The desiccated moss appears to be dead—brown, shrunken, and lifeless. This could not be further from the truth. The desiccated moss gametophores will revive upon hydration and are home to two species of invertebrate animals as hardy as the moss. The animals (bdelloid rotifers and tardigrades) live within the moss water layer and equilibrate with the ambient air as the moss dries. Incredibly, the dried moss/invertebrate “clump” can survive for years in suspended animation (Dilks and Proctor 1974).

How can any organism survive complete drying? Why don’t all organisms survive complete drying? What are the structural, molecular, biochemical, and genetic mechanisms that allow organisms to tolerate desiccation? Desiccation tolerance is a fascinating phenomenon, and the authors of this book have provided an authoritative overview of research in this field.

1.2 Diversity and Distribution of Desiccation Tolerance

Anhydrobiotes, or desiccation-tolerant organisms, have been observed among the three domains of life—Archaea, Bacteria, and Eukarya (Fig. 1.1) (reviewed

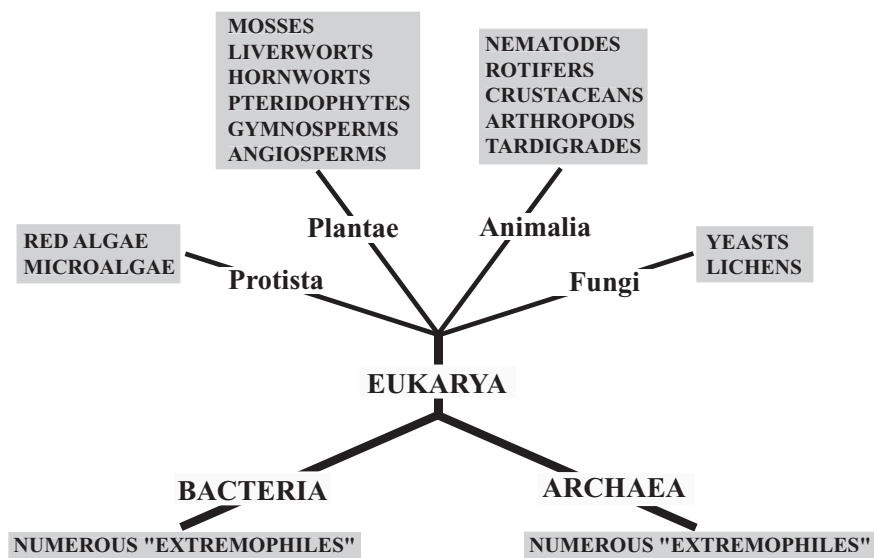


Fig. 1.1 Distribution of desiccation tolerance among the domains of life—Archaea, Bacteria, and Eukarya. Organisms within the shaded boxes have been experimentally documented to be desiccation tolerant.

in Potts 1994, 1999, Billi and Potts 2002, Alpert and Oliver 2002, Alpert 2005, 2006). In the prokaryotes, desiccation tolerance has been extensively studied in cyanobacteria (Potts 1999, Billi and Potts 2002) and is postulated to be associated with tolerance to other abiotic stress such as salinity and elevated temperature. In the Eukarya, desiccation-tolerant organisms have been characterized in each of the major lineages: protists, fungi, animals and plants (Oliver et al. 2000a, Alpert 2005, Berjak 2006) (see Fig. 1.1). The concept that is emphasized throughout this book is that *desiccation tolerance* is defined by one major criterion: What tissues or structures within an organism are tolerant? For many animals and most plants, only specialized structures such as encysted embryos (crustaceans) or seeds (vascular plants) are desiccation tolerant. However, the vegetative tissues (apparently unspecialized structures) of many organisms (epitomized by mosses and tardigrades, see earlier) are desiccation tolerant and able to survive extended periods of severe water deficit.

1.2.1 Protists, Fungi, and Animals

Desiccation-tolerant organisms are present but rare within protists, fungi, and animals.

Several species of terrestrial microalgae (Trainor and Gladych 1995), the marine red algae *Poryphyra dentata* Kjellm. (Abe et al. 2001), and most species of lichens (Kraner and Lutzoni 1999) are capable of surviving desiccation. A small number of yeasts, such as *Saccharomyces cerevisiae* Hanson (Ratnakumar and Tunnacliffe 2006), are desiccation tolerant. Surprisingly, the genetic and biochemical mechanisms of desiccation tolerance in *S. cerevisiae* are not completely characterized, although it is an active and interesting area of research.

Numerous species of nematodes (Treonis and Wall 2005), most species of bdelloid rotifers (Ricci 1998, Ricci and Capriola 2005), and many species of tardigrades (Jönsson 2005) are desiccation tolerant. Finally, specialized structures such as the embryonic cysts of brine shrimps, most notably *Artemia* spp. (Clegg 2005), and larvae of the chironomid *Polypedilum vanderplanki* Hint. (Kikawada et al. 2005) are also desiccation tolerant. Anhydrobiotic terrestrial fungi and animals are able to survive in very cold and/or dry environments. Lichens and nematodes (along with mosses, see later) are important species of both the Arctic and Antarctic fauna and flora (Lovelock and Robinson 2002, Treonis and Wall 2005).

1.2.2 Plants

Terrestrial land plants live within a “water-limiting environment” (Wood 2005). In contrast to protists, fungi, and animals, specialized desiccation-tolerant structures are both common and widespread within the land plants. A central aspect of the land plant life cycle is the production of a reproductive structure(s) capable of surviving desiccation, most notably orthodox seeds but also spores and pollen. Desiccation-tolerant reproductive structures are found within the bryophytes, pteridophytes, gymnosperms, and angiosperms (Oliver et al. 2000a). Desiccation tolerance within vegetative tissues (as seen in nematodes, rotifers, and tardigrades) is widely distributed and, with the notable exception of the gymnosperms, is present within most classes of plants (Oliver et al. 2000a, Porembski and Barthlott 2000). Desiccation-tolerant plants (i.e., plants that exhibit tolerance within vegetative tissues) are found within the bryophytes (Oliver et al. 2005, Proctor et al. 2007, Wood 2007), pteridophytes, and angiosperms (Bartels 2005, Illing et al. 2005).

An authoritative checklist of desiccation-tolerant tracheophytes was compiled by Proctor and Pence (2002). Approximately 320 species of vascular plants (less than 0.15% of the total) possess vegetative desiccation tolerance (Porembski and Barthlott 2000). They reside within 9 pteridophyte families (Adiantaceae, Aspleniaceae, Davalliaceae, Grammitidaceae, Hymenophyllaceae, Isoëtaceae, Polypodiaceae, Schizaeaceae, and Selaginellaceae) and 10 angiosperm families (Acanthaceae, Cactaceae,

Cyperaceae, Gesneriaceae, Labiatae, Liliaceae, Myrothamnaceae, Poaceae, Scrophulariaceae, and Velloziaceae). It is suggested that all members of the genera *Vellozia* (approximately 124 species) and *Xerophyta* (approximately 28 species) are desiccation tolerant. Detailed physiological analysis, however, has been limited to approximately 160 species. Several resurrection plants, such as *Craterostigma plantagineum* Hochst. (Bartels 2005) and *Xerophyta viscosa* Baker (Illing et al. 2005), have been developed as model experimental systems and are detailed in subsequent chapters.

The majority of the Bryophyta (approximately 21,000 species of mosses, liverworts, and hornworts) are postulated to be desiccation tolerant and able to survive brief desiccation of modest intensity (Proctor et al. 2007, Proctor and Pence 2002). A total of 210 Bryophyta species (approximately 1% of the total) have been experimentally determined to be desiccation tolerant (Wood 2007) and have been identified within seven classes of bryophytes: the Andreaeopsida, Bryopsida, Polytrichopsida, and Tetraphidopsida (mosses); Marchantiopsida and Jungermanniopsida (liverworts); and the Anthocerotopsida (hornworts).

Desiccation tolerance is considered by many as a key component in the evolution of green plants that allowed freshwater algae to successfully colonize the land (Oliver et al. 2000a). In this scenario, as land plants evolved more efficient mechanisms for internally transporting water, desiccation tolerance was apparently lost from vegetative tissues and retained with reproductive structures (Proctor and Tuba 2002). As vascular plants diversified, it is further hypothesized that vegetative desiccation tolerance independently reevolved several times, giving rise to the resurrection plants observed today (Oliver et al. 2000a).

1.3 A Definition of Desiccation and Desiccation Tolerance

Plants cannot maintain their cellular water content at a fixed point and are constantly losing water to the surrounding environment. The leaf water potential (ψ_w) for fully hydrated leaves of mesophytic plants ranges from -0.2 MPa to -0.5 MPa (Boyer 1970). Relative humidity (RH) of 100% corresponds to a ψ_{wv} of 0.00 MPa (Table 1.1). Air of 99.6% RH (-0.54 MPa) is in equilibrium with a typical land plant. Any further reductions in RH will generate a water gradient, thereby increasing the evaporative loss of water. Modestly dry air (e.g., 90% RH) corresponds to -14.2 MPa, and dry air (e.g., 50% RH) corresponds to -93.6 MPa.

Desiccation tolerance is defined as the ability of an organism to dry to equilibrium with dry air (50% RH and 20°C, corresponding water potential of -94 MPa) and to resume normal metabolic function on rehydration (Bewley

Table 1.1 Relative humidity and the corresponding values for water vapor, and water potential (ψ_{wv}).

Relative Humidity (%)	ψ_{wv} (MPa)
100.0	0.00
99.6	−0.54
99.0	−1.36
90.0	−14.2
80.0	−30.1
70.0	−48.2
60.0	−70.0
50.0	−93.6
20.0	−217.3
10.0	−310.8
0.00	−∞

Data are for 20°C and are calculated using the formula.
 $\psi_{\text{wv}} = (135 \text{ MPa}) \ln \left(\frac{90}{100} \right) = -14.2 \text{ MPa}$. Wood (2005), adapted from Nobel (1983).

1979, Gaff 1997, Alpert et al. 2002, Proctor and Pence 2002, Alpert 2005, 2006, Wood 2005, 2007, Proctor et al. 2007). Desiccation, unlike dehydration, is both a *process* and a *destination*. Desiccation is not the exposure of an organism to dry air. Desiccation is the complete loss of “free” water from an organism, which corresponds to less than 0.1 g H₂O • g^{−1} dry mass (10% water content or less) (Gaff 1971, Alpert 2005, 2006). Desiccation-tolerant specialized structures or desiccation-tolerant vegetative tissues survive severe water deficit and recover from the “air-dried state” within an ecologically relevant time frame (Bewley 1979). Some of the desiccation-tolerant organisms described earlier (seeds, rotifers, tardigrades, and lichens) can survive as desiccated material for decades. Practically, they must survive only until the next rehydration event.

Finally, desiccation tolerant plants can be roughly divided into two broad categories based upon the speed at which the tissues desiccate. Category 1 organisms can tolerate rapid desiccation (i.e., desiccation within a few minutes) and are comprised almost exclusively of bryophytes [as exemplified by the moss *Tortula ruralis* (Hedw.) Gaertn. (Wood et al. 2005, Wood 2007)]. Interestingly, category 1 would include lichens, rotifers, and tardigrades. Category 2 organisms/structures can tolerate desiccation only if water loss is relatively slow; this category is composed of most vascular resurrection plants, pollen, and seeds. As detailed in several chapters from this book, resurrection plants and seeds possess a host of regulated features that become established as the tissues or structures desiccate.

1.4 The Future of Research on Desiccation Tolerance

One of the most significant limitations upon crop productivity is the availability of water (Boyer 1982, Wood 2005). Agricultural regions routinely experience drought, and current models predict that rainfall patterns will be dramatically altered within the context of global climate change. Against this backdrop, agricultural production must be enhanced worldwide. The traditional demand for “food, feed, and fiber” is constantly increasing. And, the new demands for alternative energy sources and the production of biofuels will only create a greater need for the world’s limited water resources (Hill et al. 2006).

We are convinced that understanding the basic science that underlies desiccation tolerance can have a profound impact upon the “real world.” For example, the identification and characterization of sugars, polyols, and proteins associated with desiccation tolerance have allowed for the preservation and stabilization of medically important cells and cell products (Crowe et al. 2005, Kanas and Acker 2006, Potts et al. 2005). A better understanding of mechanisms controlling vegetative desiccation tolerance within bryophytes, lichens, yeast, ferns, and resurrection plants could allow for the creation of more drought-tolerant and/or water use-efficient crop plants (including agronomic, horticultural, and landscape plants)—plants that are able to thrive under more water-limiting conditions (Bartels and Sunkar 2005, Bohnert et al. 2006, Wood 2005). A better understanding of desiccation tolerance within specialized structures will provide greater insight to improving seed quality and the long-term preservation and storage of seeds, pollen, and plant propagules (Berjak 2006). Besides these, resurrection vascular plants [in particular, those native to South Africa such as *Myrothamnus flabellifolia* Welw. (Moore et al. 2007)] may have novel uses in horticulture, such as in replacing high-water use landscape ornamentals to significantly reduce the need for irrigation water in the managed landscape. As these examples reveal, there is much potential that continued research into plant desiccation tolerance could translate into new and useful applications in fields as diverse as medicine, agriculture, and landscape management.

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2 Lessons on Dehydration Tolerance from Desiccation-Tolerant Plants

Melvin J. Oliver

2.1 Introduction

Extremophiles are organisms that thrive (a relative term) in environments where conditions are such that the majority of organisms cannot survive. This is not strictly true if one is describing desiccation-tolerant plants, as other plants do grow around them, but it is certainly true that they can survive levels of dehydration, or water deficits, that the majority of plants cannot. Bewley (1979) defined *desiccation tolerance* as the ability to “revive from the air-dry state (the air being of low relative humidity), and a plant which is desiccated is one from which all available water has been lost to the surrounding dry atmosphere.” In more precise terms, we tend to define desiccation tolerance as the ability to equilibrate cellular water potential with that of moderately dry air and then resume metabolism and growth upon rehydration. In a practical sense, described by Gaff (1997) and Proctor and Pence (2002), equilibration of cells with air of relative humidity (RH) at 50% and at 20°C would lead to a corresponding water potential of -100 MPa (or $-1,000$ bars). On a water content basis, often used to describe the level of dryness of seeds (desiccation-tolerant tissues of most plants), this would translate to $0.1 \text{ g H}_2\text{O} \cdot \text{g}^{-1}$ dry mass (Alpert 2005), or about 10%. If one were to consider this an extreme and a plant that can tolerate such a cellular water potential or water content as an extremophile, then one would expect that the majority of plants could not survive such treatment. This does appear to be the case. The vast majority of plants cannot survive much beyond -10 MPa, the lowest survivable water deficit recorded (at least as far as I could discover) by a desiccation-sensitive plant is -12.1 MPa (-121 bars) for *Larrea divaricata* (a desert shrub, Cunningham and Burk 1973), and most crop plants are far more sensitive, dying at -2 to -4 MPa (Boyer 1970; Richter 1976). Desiccation-tolerant plants however, all survive a minimum of -100 MPa, and most much lower water deficits. Desiccation-tolerant bryophytes, for example, can survive drying to water deficits closer to -600 MPa ($-6,000$ bars) and still recover quickly (Oliver et al. 1993, 2005). Not only can bryophytes tolerate extreme water deficits, but they can do so for very long periods of time, up to 10 to 20 years for some species (Alpert 2000). It is with consideration of the large disparity in survivable water deficits or, more accurately, dehydration levels, between sensitive and desiccation-tolerant plants that one can justifiably label desiccation-tolerant plants as extremophiles. Desiccation-tolerant plants

also earn the distinction of being extremophiles in that when desiccated, these plants can survive extreme temperatures, from liquid nitrogen temperatures of -272°C to that of 100°C (reviewed in Alpert 2000). In fact, one could pose the question, Did the ability to dry and survive evolve as a means by which plants could survive the large temperature fluctuations that are encountered in xeric habitats and not simply as a means to tolerate dry atmospheres?

The large disparity in the ranges of tolerance to dehydration in sensitive and desiccation-tolerant plants reveals a fundamental difference between drought tolerance in sensitive species and desiccation tolerance. Sensitive species, when soil moisture becomes limiting during a drought, utilize mechanisms to retain water and to limit the amount of dehydration to a level that can be endured over a relatively short period of time. More precisely put, sensitive plants attempt to “maintain a chronic disequilibrium between wet cells and dry air” (Alpert and Oliver 2002), and when this strategy fails, they die. Drought tolerance can be described as the relative ability a plant has to maintain the disequilibrium, limit dehydration to a noninjurious level, and, in agronomic terms, maintain productivity when soil water potentials are low. Desiccation-tolerant plants, however, react to low soil moisture and dry air by initiating mechanisms that prepare the vegetative cells for dehydration and do not attempt to maintain the chronic disequilibrium between wet cells and dry air. The question remains, as discussed later in the chapter, as to whether desiccation-tolerant plants respond to mild water deficits in a similar way as sensitive plants, or do they initiate preparations for dehydration as soon as leaf water potentials (if this is the trigger) drop below some critical level? An equally pertinent question, especially with regard to strategies to improve drought tolerance in sensitive crops, is do sensitive plants utilize similar components and strategies to endure dehydration and the accompanying cellular injury; if they do, is the water deficit response we see in sensitive plants just a low level or partial expression of desiccation tolerance? I attempt to address both of these questions in the following narrative with a view to revealing what lessons have been learned from desiccation-tolerant plants (extremophiles).

2.2 Variability in Desiccation Tolerance

Although desiccation tolerance can be readily defined and experimentally separated from desiccation sensitivity, there is a great deal of variability within the phenotype itself (Alpert and Oliver 2002, Proctor and Pence 2002, Alpert 2005, Proctor et al. 2007). Most desiccation-tolerant plants can survive equilibration with air less than 1% RH (Alpert 2005), but this survival is directly influenced by the rate at which dehydration occurs in order to reach the desiccated state. Desiccation-tolerant bryophytes survive desiccation even if the rate

of dehydration is very rapid, equilibration to less than 5% RH (approximately -500 MPa) within 30 to 60 minutes (Oliver and Bewley 1997), whereas desiccation-tolerant angiosperms require a much slower dehydration rate, in many cases, longer than 24 hours (Alpert and Oliver 2002). In general, desiccation-tolerant ferns also require slow dehydration in order to survive desiccation but the spikemosses, in particular, *Selaginella lepidophylla*, can survive more rapid water loss, reaching the dried state within 5 to 6 hours (Eickmeier 1983). In some cases, vegetative desiccation tolerance can be increased both naturally and experimentally. Seasonal variations in desiccation tolerance have been reported for several bryophyte species (Proctor and Tuba 2002, Proctor et al. 2007a), a good example of which is the South African moss *Atrichum androgynum*, which becomes more sensitive to desiccation in the wetter months of the year (Beckett and Hoddinott 1997). *Lunularia cruciata*, a liverwort, switches from sensitive winter to tolerant summer stages that appear to be photoperiodic in nature and controlled by changes in lunularic acid levels (Schwabe and Nachmony-Bascomb 1963). Desiccation tolerance can also diminish as many plants become more sensitive to desiccation after prolonged periods of full hydration (e.g., Gaff 1977, Schonbeck and Bewley 1981, Kappen and Valladares 1999). The length of time that plants can remain desiccated, especially under field conditions, also varies considerably. Oliver et al. (1993) demonstrated that three desiccation-tolerant *Tortula* (Syntrichia) spp., [*Tortula caninervis*, *Tortula ruralis*, and *Tortula norwegica*] could be differentiated on the basis of their ability to survive long-term desiccation, with *T. caninervis* (a desert species) the most tolerant by this criterion. The length of time a plant can survive desiccation is not a practical measure of tolerance, however, as several desiccation-tolerant bryophytes (both mosses and liverworts) can recover after 20 to 25 years at air dryness, and many desiccation-tolerant angiosperms can survive after more than 5 years (Gaff 1977, Alpert and Oliver 2002). Viability of dried plants is also highly dependent upon the storage conditions; for example, most desiccation-tolerant bryophytes survive best and thus longer if kept equilibrated to atmospheres between 20% and 50% RH at 20°C (-100 to -400 MPa). Storage at lower RH levels leads to a quicker loss in viability (Proctor and Tuba 2002). Seeds of land plants that acquire desiccation tolerance during maturation on the parent plant (termed *orthodox seeds*) have been known to remain viable in the dried state for extremely long periods of time, the longest documented case being circa 1100 years for the seed of *Nelumbo nucifera* (Shen-Miller et al. 1995).

2.3 Hardening and Priming for Tolerance

Experimentally, the level of tolerance in a plant or plant tissue to desiccation can be increased by applying a mild dehydration treatment prior to equilibration

with dry air to generate desiccation, a process called *hardening* (Proctor and Pence 2002). Many bryophytes require as little as a few hours of exposure to 96% RH in order to exhibit an increase in desiccation tolerance (Abel 1956). Even the highly tolerant *T. ruralis* increases in tolerance by prior exposure to mildly drying atmospheres (Schonbeck and Bewley 1981). This phenomenon is not limited to bryophytes, as similar observations have been reported for the desiccation-tolerant fern *Polypodium virginianum* (Bewley et al. 1993) and for detached leaves of the desiccation-tolerant angiosperm *Borya nitida* (Gaff and Loveys 1984). It is likely that this is a universal and natural component of the desiccation tolerance phenotype in plants. Many of those who work with desiccation-tolerant plants have observed this phenomenon but have not reported its occurrence in the scientific literature. The evidence that we have, and that is discussed later in this chapter, suggests that prior and partial dehydration treatments prime the cells with protective proteins and compounds that are key components in desiccation tolerance mechanisms, so that when desiccation does occur, the cell has accumulated sufficient amounts of these compounds to avoid the cellular injury that would normally be associated with desiccation (Rascio and La Rocca 2005).

In a number of desiccation-tolerant plants, an exogenous application of the plant growth regulator abscisic acid (ABA) can mimic a predesiccation drying treatment in elevating the level of desiccation tolerance either in tissues that are sensitive, so that they become tolerant, or in whole plants, such that they can survive either faster rates of water loss or greater depths of desiccation. Beckett (1999) demonstrated that treatment of *Atrichum androgynum* gametophytes with exogenous ABA had the same effect as a 3-day partial dehydration treatment in regard to the resistance of the moss to desiccation-induced ion leakage, a measure of membrane damage. Pretreatment of fronds of *Polytrichum virginianum* increased the tolerance of this fern to desiccation such that it could survive a normally lethal rapid dehydration treatment (Reynolds and Bewley 1993). Callus tissue derived from the leaves of the desiccation-tolerant dicot *Craterostigma plantagineum* is sensitive to desiccation but, if treated for 4 days with ABA before drying it, develops tolerance (Bartels et al. 1990). Treatment of detached leaves of *Borya nitida* and *Myrothamnus flabellifolius* (a desiccation-tolerant shrub) for 48 hours permits them to survive a rapid drying treatment that is normally lethal (Gaff and Loveys 1984). In contrast, exogenous ABA treatments to leaves of the desiccation-tolerant grass *Sporobolus stapfianus* does not result in an increase in their level of desiccation tolerance (Gaff and Loveys 1994). Again, as for the hardening process, the indication is that ABA can act as a cellular signal that primes the cells such that they initiate synthesis of protective proteins and compounds that allow the cells to survive a subsequent and potentially injurious desiccation event. The ABA-induced induction of tolerance-related protein synthesis has been demonstrated directly in several species. For example,

ABA treatment of *Funaria hygrometrica* not only increases tolerance to desiccation but also results in the induction of the synthesis of a number of proteins that accumulate during drying and have been associated with tolerance in other species (Werner et al. 1991, Bopp and Werner 1993). Application of ABA to the fronds of *Polypodium virginianum* results in the synthesis of proteins that are similar to those made during desiccation (Reynolds and Bewley 1993a, 1993b), and such fronds can survive rapid desiccation. The induction of tolerance by ABA treatment of the desiccation-sensitive callus tissue of *C. plantagineum* is also associated with the synthesis of a number of proteins that are normally seen only during desiccation of attached leaf tissues (Bartels et al. 1990). ABA is more than just an experimental tool to prime cells for desiccation; it is considered to be a major component of at least one of the endogenous signaling pathways that control gene expression associated with the acquisition of desiccation tolerance and the response of desiccation-sensitive species to drought (Phillips et al. 2002, Shinozaki et al. 2003, Vitré et al. 2004a, Bartels and Sunkar 2005). This is more fully addressed in later sections of this chapter.

2.4 Evolutionary Aspects of Desiccation Tolerance in Plants

Vegetative desiccation tolerance is a trait that is present throughout the phylogeny of the land plants, from bryophytes, which are representatives of the basal most clade, to the angiosperms that represent the most recently evolved clade and the most complex of living plants (Bewley and Krochko 1982, Oliver et al. 2000, Proctor and Pence 2002) (Fig. 2.1). However, its presence is not continuous as it is absent in gymnosperms (a taxonomic group consisting of the phylogenetically distinct cycads, conifers, and gnetophytes). Oliver et al. (2005) postulate that desiccation tolerance is a trait that was present in primitive plants and was required in order for the successful colonization of dry land from fresh water that occurred roughly 470 million years ago (Heckman et al. 2001). Desiccation tolerance is not universal in modern-day bryophytes, but it is thought to be common (Proctor 1990, Richardson 1981), occurring in greater than 10% of mosses for example. Wood (2007) surveyed the bryophytes for species for which desiccation tolerance had been experimentally determined, in contrast to anecdotal observations, and found that 1% of all bryophytes could be classified as tolerant: 158 species of mosses, 51 liverworts, and 1 species of hornwort. This number will undoubtedly grow as more species are tested in the laboratory or field. Vegetative desiccation tolerance as a trait was lost during the early evolutionary events that led to the appearance of tracheophytes. The loss of vegetative desiccation tolerance at this stage in the evolution of land plants has been explained by what is termed

Postulated Evolutionary History of Desiccation Tolerance in Land Plants

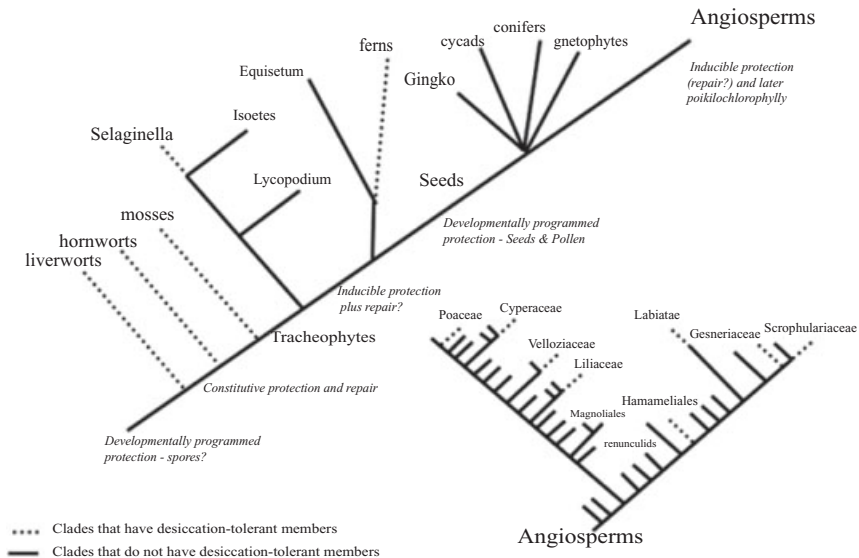


Fig. 2.1 Proposed evolutionary history of desiccation tolerance in the land plants and associated tolerance strategies (adapted from Oliver et al. 2000, 2005).

the *desiccation tolerance/productivity tradeoff* hypothesis, that high desiccation tolerance is linked to slow growth (Oliver et al. 2000, Alpert and Oliver 2002, Alpert 2006). Thus, with the evolution of internal water relations and water transport mechanisms, it is theorized that early tracheophytes abandoned vegetative desiccation tolerance in favor of the more rapid growth rates and greater competitiveness for niche selection that water retention mechanisms afforded. This hypothesis is plausible because desiccation tolerance entails metabolic costs and depends on traits likely to limit productivity (Alpert 2006). The genetic ability to tolerate desiccation, however, was not lost from the land plant lineage, as early tracheophytes were still able to produce desiccation-tolerant propagules. This is underscored by the fact that vegetative desiccation tolerance “re-evolved” in both the *Selaginellas* (spike mosses) and the *Leptosporangiate* ferns (Oliver et al. 2000, 2005). This also indicates that the genetic capability for desiccation tolerance was still available to the ancestral forms of these groups and could be redirected to ensure survival of the sporophytic generation in extreme environments. Desiccation tolerance in propagules persisted in the land plant lineage and evolved from the apparently simple mechanism seen in spores to the more complex developmentally controlled mechanism of tolerance that first appeared early on in

the evolution of seeds as vehicles for generational dispersal. As plants increased in complexity and adapted to the myriad of ecological niches that dry land offered, vegetative desiccation tolerance again became advantageous in some plant lineages and thus “re-evolved” again. Within the angiosperms, vegetative desiccation tolerance has reappeared in at least 10 phylogenetically independent lineages and thus by inference from 10 independent evolutionary events (Proctor and Pence 2002, Oliver et al. 2005). Of interest is that none of these events occurred in the basal dicots; however, there were six events in the eudicots and four in the monocots. To date, approximately 330 species of vascular plants have been designated as desiccation tolerant; of these, about 233 are angiosperms (Proctor and Pence 2002). As more cases of vegetative desiccation tolerance are discovered in the angiosperms, as expected for the Poaceae and Cyperaceae, it will be interesting to see if the number of independent “re-evolutions” of this important trait will increase.

As the details of the evolution of desiccation tolerance in plants unravel, we are beginning to understand the various mechanisms or strategies, and the components that go together to effect their success, which plants utilize to acquire the ability to dry without dying.

2.5 Mechanistic Considerations

In a landmark review of the physiological aspects of desiccation tolerance in plants, J. Derek Bewley (1979) proposed the currently accepted theory that desiccation tolerance is primarily an inherent property of the cellular contents (protoplasm). Bewley (1979) defined what the properties of a desiccation-tolerant protoplasm must encompass: it must (a) limit damage to a repairable level, (b) maintain cellular integrity in the dried state, and (c) upon rehydration, rapidly mobilize repair mechanisms to counter damage suffered during drying or rehydration. Mechanistically, this translates into a combination of the protection of cellular structures and components both during drying and in the dried state coupled with an active cellular repair activity during and following rehydration (Bewley and Oliver 1992). Thompson and co-workers, (Platt et al. 1994, Thompson and Platt 1997), based on freeze fracture and freeze substitution electron microscopy of dried and drying *T. ruralis* (freeze-fracture only) and *Selaginella lepidophylla* (a spikemoss), added a further requirement that physical cell order is maintained both during drying and in the dry state. This conservation of cell order requires a high degree of effective packing and shape fitting of cellular constituents driven by the compaction forces of dehydration. Proctor et al. (2007b) and Pressel 2007 (reported in Proctor et al. 2007) provided evidence that the microtubular cytoskeleton is perhaps a key component in the control of cellular order during both desiccation

and rehydration. Alpert and Oliver (2002) has previously suggested that desiccation tolerance must also require an orderly shutdown of metabolism during drying to avoid the possibility of a build-up in toxic intermediates and the generation of reactive oxygen species (ROS), a major stress associated with desiccation (Smirnov 1993).

2.5.1 Cellular Dehydration

How successful are desiccation-tolerant plants in preventing both dehydration- and rehydration-induced cellular damage, and how much repair is needed to allow for an efficient recovery from drying? In any discussion of cellular damage as a result of desiccation, one has to take into account that, as mentioned previously, the rate at which desiccation occurs has a great impact on the integrity of the cells that are dehydrating. In addition, however, damage may not be visible in dried cells; as I will discuss, that does not mean that damage did not occur as much of the types and mechanisms of damage ascribed to drying of biological tissues takes place at the molecular level (e.g., protein or DNA modifications). However, for a more detailed review of the possible types of cellular damage and how they come about, I refer the reader to the excellent review on this topic by Walters et al. (2002).

Much of the early work with regard to ultrastructural observations of desiccated plant cells centered on the desiccation-tolerant bryophytes and, even given the limitations of the technology available to observe dried specimens, offered some interesting insights (reviewed by Oliver and Bewley 1984). Some of the more interesting studies relate to the use of Nomarski optics, a form of interference-contrast microscopy, which does not require the use of fixatives that might allow partial or complete rehydration of the cell (the main source of artifact in much of the earlier work). Tucker et al. (1975) demonstrated that leaf cells of *T. ruralis* underwent extensive collapse upon dehydration with the protoplasm condensing at the proximal and distal ends, leaving the center of the cells empty. Cytoplasmic bridges that run along the abaxial, adaxial, and lateral sides of the cells join the two areas of condensed cytoplasm. Chloroplasts appear smaller and more spherical than in hydrated cells and tend to be at each end of the cells; nuclei appear normal, however. Unfortunately, this type of microscopy allows for much less detail to be seen compared with electron microscopy. What this technique did reveal is that not all bryophytes dry in the same way. Moore et al. (1982), again using Nomarski optics, determined that cells of *Barbula torquata* and *Triquetrella papillata*, rather than allowing the entry of air into the cells, shrank to 50% to 70% of their original volume, resulting in invagination of the cell walls. This variation in how cells dry has also been demonstrated for desiccation-tolerant angiosperms. Farrant (2000), using the more modern techniques of freeze

substitution, compared dried leaf cells from three species of desiccation-tolerant angiosperms: *Xerophyta humilis*, *Craterostigma wilmsii*, and *Myrothamnus flabellifolius*. In all three species, the vacuoles within the cells were filled with nonaqueous materials, presumably osmoprotectants. However, the results of this differed for each plant. In *X. humilis*, the vacuoles filled most of the cytoplasm, which prevented plasmolysis and cell wall collapse. *C. wilmsii* maintained small vacuoles and relied upon extensive invagination and folding of the cell wall to prevent plasmolysis. *Myrothamnus flabellifolius* exhibited a pattern that was somewhat in between these two scenarios in that some vacuolation occurred along with some cell wall folding but neither was sufficient to prevent shrinkage of the plasmalemma away from the cell walls (although the membrane was apparently undamaged in the process). Obviously, there is more than one way to deal with rigors of water loss from cells but, as will become evident, each way is apparently effective in allowing for an ordered and controlled packing of the cytoplasm as well the maintenance of membrane integrity, at least to repairable levels.

With the advent of more sophisticated technologies for observing the conditions of dried plant cells a more detailed picture emerged. Platt et al. (1994) used freeze-fracture technology to observe membrane structures in dried *T. ruralis* and *Selaginella lepidophylla* as a way of avoiding the possibility of artifacts due to partial rehydration and the use of fixatives. In both species, the cell membranes remain as intact bilayers containing normally dispersed intramembranous particles when in the air-dried state. The structural organization of the organelles is also maintained in both species, both thylakoid and cristae membranes appear intact, and no areas of disrupted bilayer organization were detected in any of the cell membranes. At least for these two species, it does not appear that desiccation, rapid in the case of *Tortula* and slow for *Selaginella*, causes observable physical damage, especially with regards to the integrity of cellular membranes. Similar conclusions have been drawn from the use of freeze-fracture to investigate membrane structure in dried seeds (Thompson and Platt-Aloia 1982, Bliss et al. 1984, Platt-Aloia et al. 1986). Farrant (2000), as mentioned previously, used cryofixation and freeze-substitution to examine slowly dried leaf cells of several desiccation-tolerant angiosperms and reports similar findings regarding the integrity of cell membranes and organelles in the dried state, even though each species had a characteristic way of dealing with the mechanical stresses of water loss. We do know, however, that cell membranes are affected by desiccation. Buitink et al. (2000) demonstrated that tolerant tissues differ from sensitive tissues in the partitioning of amphiphilic substances into membranes from the cytoplasm, a process that is linked to increases in membrane leakage (Golovina et al. 1998). Pressel et al. (2006) used anhydrous fixation techniques to demonstrate that although cells of slowly dried *Polytrichum formosum* (a desiccation-tolerant moss) did undergo extensive changes, most notably, a loss

of endoplasmic microtubules and the replacement of the endoplasmic reticulum with membranous tubules, there was little evidence of actual damage. Proctor et al. (2007b) reaffirmed the lack of structural damage during slow dehydration of the leaf cells in this species. The inference that can be taken from such studies is that cell order is maintained in dried cells of desiccation-tolerant plants and that there is little to no observable damage. Whether this is true for sensitive vegetative tissues that have been air-dried is still a matter of debate, as in some cases cell order appears to be retained (e.g., in radicles of developing desiccation-sensitive jackfruit seeds) (Wesley-Smith 2001), and in others order appears disrupted as in the case of detached and desiccation-sensitive leaves of *Sporobolus stapfianus* (Quartacci et al. 1997) and for chloroplasts of *Pisum sativum*, within which the thylakoids, although still stacked, appear blistered (Sherwin and Farrant 1996). It is clear, however, that cellular damage occurs in both desiccation-sensitive and -tolerant plants if they are maintained at intermediate water potentials (Walters et al. 2001), and so it appears that less is better when it comes to water loss and visible cellular damage.

2.5.2 Cells in the Dried State

Cells in the air-dried state are not inert; chemical reactions do occur, and with them comes the potential for and certainty of cellular damage. Water loss from cells creates an increase in viscosity of the cytoplasmic milieu that, as dehydration advances, moves toward the establishment of a solid matrix. As this process progresses, the concentration of cellular metabolites that could react with one another increases, but at the same time, the increase in viscosity slows the molecular motion that would allow reactions to occur. Walters et al. (2002) point out that the degree by which cells are damaged during dehydration and in the dried state is determined by the treatment duration, the concentration of metabolites, and the physical barriers (compartments and increased viscosity) that separate metabolites and prevent reactions from occurring. These parameters also affect the critical moisture level that a cell can survive. That damage does occur while plants are in the air-dried state is ultimately evident from the loss of viability that is associated with prolonged periods of dryness in the field and long-term storage in the laboratory. Equally obvious is the fact that the ability to limit such damage varies among desiccation-tolerant plants; some can remain viable for many years in the dried state, while others remain viable for only months (see earlier and Proctor and Pence 2002). Ability to limit such damage also varies with the prevailing conditions in the field and within storage (Alpert and Oliver 2002, Alpert 2005). Damage occurs because of the proximity of cellular components and the reactive groups that are brought together by this closeness, but by far the most

damaging process is the production of ROS and free radicals, both of which are generally byproducts of continued respiration and uncoupled light harvesting activities within the chloroplasts of dried cells, the latter being the most prolific source. Light generally increases the amount of desiccation-induced oxidative damage in plant tissues (Smirnoff 1993), and this is of considerable importance for many desiccation-tolerant plants, as they generally inhabit environments that require long periods of dryness in full sunlight. Presumably, it is this reality that generated the selection pressure that led to the evolution of poikilochlorophyllous desiccation-tolerant species. This group of desiccation-tolerant plants, mainly monocots, lose their chlorophyll and totally dismantle their photosynthetic apparatus during desiccation (Proctor and Tuba 2002). The thylakoids within the chloroplasts of these plants are taken apart in an ordered deconstruction process during drying and are resynthesized in an equally ordered process upon rehydration. Tuba et al. (1998) do not equate this process with any form of dehydration-induced damage, seeing this as an organized protection mechanism, but rather as a new, and in fact the most recently evolved, strategy for desiccation tolerance.

As in all plants, ROS and free radicals are extremely toxic and react with cellular components of all types, including protein, lipids, and nucleic acids (Smirnoff 1993), and, as will be discussed, desiccation-tolerant plants have mechanisms by which the influence of these compounds is controlled. Under most conditions, once ROS and free radicals have reacted with a component, they cause permanent damage, denaturing enzymes, membranes, and chromosomes (reviewed by Walters et al. 2002). Lipid peroxidation as a result of ROS activity is particularly noticeable in that it decreases the fluidity of membranes, interfering with their ability to maintain selective transport upon rehydration (McKersie et al. 1988, 1989).

2.5.3 Rehydration and Cell Structure and Recovery

Any damage that occurs either during desiccation or in the dried state is manifested when the cells rehydrate; indeed, because rehydration itself can cause damage (Osborne et al. 2002), it is questionable as to when damage to cells actually occurs. Most desiccation-tolerant angiosperms (Sherwin and Farrant 1996) and orthodox seeds (Osborne et al. 2002) are capable of controlling and slowing the rehydration process, allowing for order in an otherwise chaotic event. Bryophytes, however, have no such capability and rehydrate almost instantaneously when water is added. The first indication that there is an alteration in cellular structure as a result of desiccation is the leakage of solutes from the protoplasm to the external medium during rehydration of dried plant cells. This leakage is transient in desiccation-tolerant plants, and the extent to which solutes are lost depends upon the rate at which the cells were dried

subsequent to rehydration: the faster the speed of dehydration, the greater is the leakage of solutes upon rehydration (Bewley and Krochko 1982, Oliver and Bewley 1984, Oliver et al. 1993). It is generally accepted that such leakage of solutes from rehydrating cells is the result of an increase in permeability that occurs as the lipid bilayers of membranes pass through a phase transition that is driven by the hydration state of the membrane: the drier the membrane, the greater is the elevation of the phase transition temperature and the greater is the likelihood that a transition from liquid crystalline to gel phase will occur at physiological temperatures (Crowe et al. 1992). Presumably, rapid desiccation, using very dry atmospheres, causes an increase in the packing of the polar head groups within the lipid bilayers above that seen in slow desiccation, resulting in a greater elevation in the phase transition temperature, which in turn increases the extent to which membrane phase changes occur. Alternatively, it is also possible that rapid desiccation does not allow for protective measures that stabilize the membranes to be fully deployed.

More recently, the phase transition theory for rehydration-induced cell leakage has been challenged, and a new hypothesis has emerged from studies on dehydrating and rehydrating pollen (Hoekstra et al. 1997, 1999, Golovina et al. 1998, Buitink et al. 2000). During dehydration, endogenous amphipathic substances have been shown to partition from the aqueous cytoplasm into pollen membranes, and it is this process and the presence of these amphipaths in the membrane that cause imbibitional leakage (Golovina et al. 1998). Furthermore, upon rehydration, the amphipathic substances move out of the pollen membranes and leakage stops. This hypothesis could explain how transient leakage can occur through an intact membrane. Buitink et al. (2000) demonstrated that the movement of amphiphilic compounds into membranes also occurs in imbibing radicles of peas and cucumbers. Golovina et al. (1998) speculate that amphipaths may have antioxidant properties that protect membranes from damage by free radicals and, if so, imbibitional or rehydration driven leakage may be a necessary tradeoff for protection.

Observations of cellular damage at the fine structure level in rehydrated tissues are relatively few, but what is clear is that damage can occur in desiccation-tolerant plant cells but, as stated previously, it is highly dependent upon the speed of desiccation, the depth of desiccation, and the species. Nowhere is this better illustrated than in a comparison of the effect of drying rates on the cellular structure during both dehydration and rehydration of three desiccation-tolerant species of angiosperms: *C. wilmsii*, *X. humilis* (a poikilochlorophyllous monocot), and *Myrothamnus flabellifolius* (Sherwin and Farrant 1996, Farrant et al. 1999). For all three species, slow dehydration resulted in the ordered collapse of cells, although each in its own particular fashion and no obvious indication of cell damage, and all survived rehydration, albeit at different rates of recovery. In *M. flabellifolius*, a woody shrub, recovery was slowed primarily because of the time needed to "refill" the xylem vessels and

deal with possible embolisms; however, these plants did have a significant delay in the resumption of photochemical activity that the authors attribute to a lack of continuity between thylakoids that needs to be repaired before full activity can be restored (Sherwin and Farrant 1996). Other than this minor damage, slow dehydration, coupled with the somewhat controlled rehydration that these plants experience, is relatively benign and the protective measures that these plants employ are sufficient to limit damage. When the drying rate is increased, to occur within 6 to 8 hours, which is rapid for these plants, the scenario is very different. For both *X. humilis* and *M. flabellifolius*, the rapid drying treatment was lethal; cellular damage in both species was extensive and included extensive rupturing of the plasmamembrane and degeneration of the cytoplasm. Some of this damage clearly occurred during the drying phase and was exacerbated by rehydration. Desiccation was achieved at a rate that was sufficiently fast enough to prevent these two plants from employing both mechanisms to retard water loss and to protect the cells from drying-induced damage. In *X. humilis*, this was clearly evident by the observation that the normally controlled dismantling of the chloroplast and photosynthetic apparatus that occurs during slow drying did not occur and subsequent rehydration-induced photochemical activity did not recover (Farrant et al. 1999). *C. wilmsii*, however, did survive the rapid rate of drying and appeared unaffected, at least on an ultrastructural level, by the more stressful removal of water, and its tissues recovered at similar rates to those that had been slow-dried. Clearly, the survival of *X. humilis* and *M. flabellifolius* depends upon their ability to retard water loss so as to allow protection mechanisms time to establish and operate. In nature, one assumes that they can successfully achieve this and flourish, but it would also be reasonable to assume that occasionally they have to survive conditions that stretch their capabilities and that they have to have the ability to repair the damage that ensues. *C. wilmsii*, however, does not appear to rely heavily upon the ability to retard water loss and perhaps, as I discuss later in the chapter, this species has adopted a strategy that involves a constitutive level of cellular protection so that it does not have to put such a system in place should drying rates increase.

The cellular consequences of desiccation for bryophytes are also manifested upon rehydration, and for these plants rehydration is almost instantaneous. When water enters the rapidly dried cells of *T. ruralis*, the condensed cytoplasm rapidly expands to fill the cavity that formed as a result of dehydration (Tucker et al. 1975). In the initial stages, the organelles swell and take on a globular shape; although difficult to discern using Nomarsky optics, Tucker et al. (1975) report that the chloroplast thylakoid stacks appear to be disrupted. At 5 minutes after rehydration, electron microscopy after fixation of cells confirms that chloroplasts are swollen and thylakoid stacks are disrupted, although it does appear that the chloroplast membranes remain intact (Tucker et al. 1975, Oliver and Bewley 1984). Mitochondria also swell, and the

inner membrane structures appear dissipated. Such events seem common for desiccation-tolerant mosses in the few minutes following rehydration (Oliver and Bewley 1984), and in all cases normal cellular structure is achieved within 24 hours. It has been suggested that such cellular observations are artefacts caused by the fixation process in preparation for microscopy (Wesley-Smith 2001). Such explanation seems, however, very unlikely as fixation occurred 5 minutes after rehydration, when cells were fully hydrated and hydrated controls show no such abnormalities. In addition, chloroplast swelling was clearly evident by Nomarsky optics, and dried cells of desiccation-sensitive species exhibit identical structural abnormalities upon rehydration as those seen for desiccation-tolerant mosses, but in the case of sensitive species, the cells do not recover and they die (Bewley and Pacey 1978, Krochko et al. 1978).

Rehydration of slow-dried desiccation-tolerant bryophytes, as for slow-dried angiosperms, appears to have no or little observable affect on cellular fine structure. Proctor et al. (2007) demonstrated this for the desiccation-tolerant moss *Polytrichum formosum*. Using nonaqueous fixation techniques and moss that had been slowly dried to equilibrium with ambient air at 40% to 50% RH, these researchers were able to demonstrate that the fine structure of the moss was undamaged by desiccation and that, upon rehydration, not only did the appearance of the cells rapidly return to the predesiccated state but also both photosynthesis and respiration recovered rapidly, reaching predesiccation levels at 24 hours postrehydration. Furthermore, the initial recovery of photochemical activity and respiration did not require protein synthesis. Pressel et al. (2006) reported similar findings for the leptoids, specialized parenchymal cells, involved in sucrose transport, of *Polytrichum formosum* under the same drying conditions. These authors also demonstrate a recovery to the predesiccation state within 12 to 24 hours following rehydration and, interestingly, provide strong evidence for a key role of the microtubular cytoskeleton in the ordered collapse of cells and in the rapid cellular recovery process.

2.6 Components of Cellular Protection

It is clear that for all desiccation-tolerant plants, the protection of cellular constituents from the loss of water and the tight cytoplasmic packing that is associated with dehydration is of paramount importance for survival of desiccation. How that protection is achieved is still an area ripe for scientific investigation, but considerable strides toward understanding what the components of cellular protection mechanisms are and what their individual roles are in the process have been made over the past decade or so. As is often the case, agricultural and economic concerns drive scientific inquiry into plant processes, and the study of desiccation tolerance in plants is no exception. Much of what

we have learned concerning the actual components of cellular protection mechanisms during desiccation has been derived from the study of seeds, in particular the stages of maturation that immediately precede and encompass the transition from storage reserve deposition into the quiescent state that accompanies a developmentally programmed desiccation event (Bewley and Black 1994). Among the metabolic changes that take place just prior to or during the drying event is the synthesis of specific proteins and sugars, which have long been postulated to form the basis for a series of overlapping protective mechanisms that limit damage to cellular constituents (Bewley 1979, LePrince et al. 1993, Oliver and Bewley 1997). These two components, along with the aforementioned ROS protection components, have since been widely implicated as critical for desiccation tolerance in all plant cells including vegetative cells (Ingrams and Bartels 1996, Oliver and Bewley 1997, Scott 2000).

Over the past few years, a number of excellent and comprehensive reviews have discussed the various components of cellular protection processes and their suggested roles and activities in achieving a state of stability for cellular contents during desiccation (Close 1996, Ingram and Bartels 1996, Smirnov 1998, Cumming 1999, Hoekstra et al. 2001, Buitink et al. 2002, Apel and Hirt 2004, Mittler et al. 2004, Wise and Tunnacliffe 2004, Bartels and Sunkar 2005). For this reason, my discourse on this topic will be relatively brief and pointed; for a more comprehensive picture, I refer the reader to the aforementioned materials.

2.6.1 *Proteins*

The most prominent group of proteins associated with cellular protection during desiccation are the late embryogenesis abundant (LEA) proteins, first described by Galau et al. (1983), as proteins that are developmentally induced during the postabscission stage of embryogenesis in cotton. These proteins are also environmentally induced in cotton embryos by desiccation or culture in the presence of ABA or an osmoticum (Hughes and Galau 1991). Since these initial reports, the LEA proteins have proved to be ubiquitous, occurring in all orthodox seeds and in vegetative tissues in association with responses to dehydration, osmotic, and chilling stresses (Close 1996, Cumming 1999, Bartels and Sunkar 2005). LEA proteins have been segregated into at least five major groups, and all have functions that are, as of yet, largely unknown (Cumming 1999). All LEA proteins are highly hydrophilic, and all are very stable as evidenced by their resistance to the denaturing effects of boiling (with the exception of group 5 LEAs). Each of the five groups are characterized by particular structural motifs—for example, Group 1 LEAs are characterized by a 20-amino acid motif and are represented by the wheat EM protein, the first

LEA identified (Cumming and Lane 1979), and Group 2 LEA proteins (also called dehydrins) are characterized by a 15-amino acid motif, the K-segment, a stretch of serine residues and a conserved motif near the N terminus of the protein (Close 1997). Most of the LEA protein groups have been identified in many different plants. It is because of the association of all of the LEA proteins with the desiccation phase of orthodox seed maturation that the LEA proteins are assumed to be important in the establishment of protoplasmic desiccation tolerance. The association, and hence the inference of causality, is well supported—for example, ABA treatment of immature barley embryos results in both the accumulation of LEA proteins and acquisition of tolerance (Bartels et al. 1988). When vegetative tissues are subjected to water deficits and ABA treatments, LEA proteins, not normally present in hydrated and untreated tissues, accumulate to significant levels (Bartels and Sunkar 2005). In vegetative tissues, it is mainly the Group 2 and 3 LEA proteins that accumulate in response to both dehydration and ABA (Close 1996). The role of ABA in the induction of LEA protein synthesis was elegantly demonstrated in developing *Arabidopsis* seeds. ABA-deficient (*aba*) and ABA-insensitive (*abi3*) double-mutants of *Arabidopsis* seeds do not dry on the parent plant, do not tolerate desiccation, and lack several LEA proteins (Koorneef et al. 1989, Meurs et al. 1992). These and similar observations suggest that LEA proteins serve as protective molecules enabling cells to survive a certain level of dehydration (Hand et al. 2006). Moreover, it appears that desiccation tolerance depends on a relatively high concentration of a number of different LEA proteins simultaneously expressed in response to dehydration (Oliver and Bewley 1997).

Many mechanisms have been proposed for how LEA proteins protect cells during dehydration, including hydration buffering, ion sequestration, direct protection of other proteins or membranes, chromatin structure, or renaturation of unfolded proteins (Close 1996). In vitro studies support a role for LEA proteins in the prevention of protein aggregation during desiccation and freezing (Goyal et al. 2005), and molecular models suggest LEA proteins may form cytoskeletal filaments that could stabilize cellular structure during drying (Wise and Tuncliffe 2004). Wolkers (1998) also suggests that LEA proteins may act as anchors in a structural network that stabilizes cytoplasmic components during drying and in the dried state. These possibilities are all linked to a role in cellular protection during water loss or cell maintenance in the dried state.

The small heat shock proteins (HSPs) may also play a role in cellular protection during desiccation. Like the LEA proteins, small heat shock proteins (*Hsps*) accumulate in maturing seeds of many plant species (Vierling 1991, Wehmeyer et al. 1996) prior to desiccation. Small HSPs are constitutively expressed in the leaves of *C. plantagineum* and accumulate to higher levels during desiccation (Alamillo et al. 1995). This is significant because it is only in seeds that constitutive expression of small HSPs occurs, and as I discuss

later, the mimicking of the pattern of gene expression of seeds in vegetative tissues has evolutionary significance (Illing et al. 2005, Oliver et al. 2005). Exogenous ABA also induces both the expression of small HSPs and the acquisition of desiccation tolerance in the desiccation-sensitive callus tissue derived from leaves of *C. plantagineum* (Alamillo et al. 1995). The inference from these studies is that it is the chaparonin-like activities of small HSPs that may help maintain protein structure under denaturing conditions, such as desiccation, because cellular dehydration has long been thought to impact cellular function and viability by compromising macromolecular structures, in particular, membranes and proteins.

2.6.2 Sugars

The accumulation of soluble sugars has long been correlated with the acquisition of desiccation tolerance in plants and other organisms (Crowe et al. 1992, Vertucci and Farrant 1995, Scott 2000, Phillips et al. 2002). Orthodox seeds, pollen, and most plants that accumulate soluble sugars in response to desiccation utilize the disaccharide sucrose. In *C. plantagineum*, 2-octulose stored in the hydrated leaves is converted to sucrose during drying to such an extent that in the dried state it composes about 40% of the dry weight (Bianchi et al. 1991, Bernacchia et al. 1996). Sucrose also accumulates in *Sporobolus stapfianus* and *Xerophyta viscosa* during desiccation, which is associated with a significant increase in leaf hexokinase activity (Whittaker et al. 2001). In desiccation-tolerant bryophytes, sucrose is the only free sugar available for cellular protection during desiccation (Smirnoff 1992). In the desiccation-tolerant mosses *T. ruralis* and *Tortula ruraliformis*, sucrose levels are maintained at approximately 10% of the dry mass of the gametophytes of these plants (Bewley et al. 1978, Smirnoff 1992), a level that is sufficient to offer membrane protection during drying (Straus and Hauser 1986). The level of sucrose in the gametophytes does not change during desiccation or rehydration in the dark or light (Bewley et al. 1978), inferring that for these plants a constitutive level of protection is paramount to their ability to respond to desiccation. The current hypothesis is that sugars protect the cell either via the formation of a biological glass (a supersaturated liquid with the mechanical properties of a solid that prevents the crystallization of cellular solutes) or by maintaining hydrogen bonds within and between macromolecules (the water replacement theory), thus stabilizing their structure (e.g., membranes) (Hoekstra et al. 2001). It is quite likely that both scenarios contribute to desiccation tolerance. Sucrose has long been associated with glass formation, and it is currently thought that LEA proteins are also required for the vitrification process (Buitink et al. 2002). In in vitro experiments, trehalose, a nonreducing

disaccharide of glucose, has been shown to stabilize protein structures (Crowe et al. 1992).

Other sugars also accumulate in desiccation-tolerant tissues and in seeds, the principal ones being the oligosaccharides stachyose and raffinose (Bewley and Black 1994). The presence of these sugars has also been correlated with seed longevity (Hoekstra et al. 1994, Horbowicz and Obendorf 1994). Trehalose has also been shown to contribute to desiccation tolerance in both yeast and nematodes but is not often found associated with desiccation tolerance in plants. Notable exceptions are the spikemoss *Selaginella lepidophylla*, where trehalose accumulates to relatively high levels during drying (Adams et al. 1990, Iturriaga et al. 2000), and a North American *Sporobolus* sp. (Iturriaga et al. 2000). Sugars contribute to a significant proportion of the dry weight of *Selaginella lepidophylla* tissues, and their concentration varies with hydration states (Adams et al. 1990, Figueroa et al. 2004). Trehalose, however, is either absent or present in only small amounts in other resurrection plants such as *Myrothamnus flabellifolius* and *S. stapfianus* (Bianchi et al. 1993, Drennan et al. 1993, Albini et al. 1994).

2.6.3 Reactive Oxygen Species Scavenging Pathways

As discussed previously, during dehydration, ROS and free radicals (e.g., singlet oxygen, hydroxyl radicals, hydrogen peroxide, and superoxide anions) increase in plant cells and tissues (Smirnov 1998, Apel and Hirt 2004). ROS and free radicals are primarily generated in the chloroplast by the Mehler reaction and the antenna pigments, and their main effect is to inhibit the repair of damage to photosystem II and synthesis of D1 protein (Allen 1995). Under conditions that limit CO₂ fixation, such as dehydration, the synthesis of ROS and free radicals is enhanced, even more so if there light is present (Asada and Takahashi 1987). In C₃ plants, photorespiration, another source of ROS, can also be activated if CO₂ fixation is inhibited. ROS and free radicals cause damage to cells via protein denaturation driven by the oxidation of protein sulfhydryl groups, pigment loss and photosystem damage, lipid peroxidation, and free fatty acid accumulation in membranes (McKersie 1991, Smirnov 1993).

The generation of antioxidants and the establishment of a reactive oxygen scavenging system, involving such enzymes as superoxide dismutase, the ascorbate-glutathione cycle and catalase, counter ROS activity in plants. The efficiency of these systems is closely related to an increased resistance to abiotic stresses, in particular, dehydration (Smirnov 1998, Kranner et al. 2002). This was elegantly demonstrated for the desiccation-tolerant shrub *Myrothamnus flabellifolius*, whose ability to resurrect from dryness can be

directly correlated to the state of its antioxidant defence system (Kranter et al. 2002). The plant resurjects normally if kept in the dried state for 4 months. At 8 months, when antioxidants (i.e., ascorbate, tocopherol, and glutathione) have been depleted, the plants die when watered. A dehydration-induced increase in the activity of antioxidant enzymes appears to be a general response for all desiccation-tolerant angiosperms (Farrant 2000). In bryophytes, oxidative inactivation of sulfhydryl-containing enzymes during drying has been reported for several desiccation-tolerant mosses (Stewart and Lee 1972), indicating cellular damage in these plants as a result of dehydration. Experimentally, this damage can be alleviated by incubation with reduced glutathione (GSH). Stewart and Bewley (1982) recorded a decrease in lipoxigenase activity during desiccation, suggesting a protective mechanism inherent in desiccation-tolerant species. On the other hand, Guschina et al. (2002) demonstrated that peroxidation of both phosphoglycerides and galactosylglycerides increased by 70% during dehydration of the desiccation-tolerant moss *Atrichum androgynum*. Obviously, the various capabilities, efficiency, and strategies for dealing with desiccation-induced ROS vary from species to species. This is clear from the findings that slow desiccation of *T. ruralis* results in the oxidation of the cellular GSH pool to approximately 30% of hydrated levels (Dhindsa 1987), indicating a decreased ability of the moss to withstand oxidative injury in the dried state. Desiccation of the tolerant moss *T. ruraliformis* does not result in a loss of GSH (Seel et al. 1992b), but the level of the antioxidant ascorbate does decrease during drying.

As mentioned earlier, light generally increases ROS production and consequently the amount of desiccation-induced oxidative damage in plant tissues increases (Smirnoff 1993). For example, Seel et al. (1992a) showed that the desiccation-sensitive moss *Dicranella palustris* exhibited greatly increased lipid peroxidation following desiccation, whereas the tolerant *T. ruraliformis* did not. Under high light conditions (1100 to $1200 \mu\text{mol m}^{-2} \text{sec}^{-1}$), oxidative damage and loss of chlorophyll during drying of *Dicranella palustris* increased and recovery was prevented, but light made little difference to either oxidative damage or recovery from desiccation in *T. ruraliformis*. Photoprotective mechanisms that minimize the production of ROS and detoxify those that form are important in desiccation-tolerant plants. They include thermal energy dissipation mediated by the xanthophyll cycle and antioxidation via a complex array of enzymes and redox molecules, including the GSH and ascorbate systems, which convert superoxide to water (Foyer et al. 1994, Smirnoff 1998, 2005, Logan 2005).

The ability of plants to resist cellular damage can be enhanced by overexpressing enzymes of antioxidant pathways in transgenic plants (Allen 1995); the most notable is overexpression of manganese superoxide dismutase (MnSOD) in alfalfa (McKersie et al. 1996), which generated drought-stress tolerance in the field.

2.6.4 Cell Wall Components

Cell wall composition undergoes significant changes during drying in many of the desiccation-tolerant angiosperms (Vicré et al. 2004a). Dried leaves of *C. wilmsii* have lower glucose content and a higher xyloglucan substitution rate (Vicré et al. 2004b). Cell walls also increase in extensibility during drying and recovery, correlating with expression of three alpha-expansin genes in *C. raterostigma lantagineum* (Jones and McQueen-Mason 2004). Presumably, these changes in cell wall properties relate to the strategy that these plants use to limit mechanical stresses during dehydration by allowing their cell walls to invaginate and fold.

2.7 Survival Strategies for Dehydration Extremophiles

Desiccation-tolerant plants appear to have two distinct strategies for responding to, preparing for, and surviving desiccation: constitutive protection coupled with rehydration-induced repair and induced cellular protection coupled with rehydration-induced repair. These two strategies do appear to separate along phylogenetic lines, with the former more widely representative of the less complex clades such as the bryophytes and the latter with the more complex tracheophytes such as the angiosperms. The lines of division are not absolute, as will be discussed, and as we learn more and investigate more desiccation-tolerant plants in detail and depth, the lines will blur even more, but as a generality it tends to hold at this time. The two strategies also dictate, to a large extent, the speed of drying a desiccation-tolerant plant can withstand and how quickly it can recover (obviously at one time, the rate of drying a plant would experience in its habitat would have dictated which strategy the species evolved to survive). Those plants that in large measure use a constitutive cellular protection strategy are always prepared for a dehydration event and thus can weather a rapid loss of water from their tissues (minutes in the case of bryophytes). Plants that induce cellular protection processes when dehydration occurs have to have time to establish and acquire desiccation tolerance and thus can survive only slow drying. It was a combination of the apparent division of plants phylogenetically with regard to their strategy for desiccation tolerance coupled with the closely fitted ability to survive rapid desiccation that led Oliver et al. (1998, 2000) to classify desiccation-tolerant plants into two groups: the fully desiccation-tolerant plants, which can survive desiccation at any speed, and the modified desiccation-tolerant plants, which require time to prepare for desiccation (the term “modified” being used in an evolutionary context indicating a derived phenotype). This classification is still valid, although, as with all such anthropomorphic delineations, some

exceptions are being uncovered, such as *C. wilmsii*, an angiosperm that appears to use constitutive cellular protection for its mode of desiccation tolerance (Farrant et al. 1999). Finally (for this preamble), as with all biological processes that are environmentally driven, there is a great deal of phenotypic variation, and desiccation tolerance is no exception. Individual species will always have their own particular nuance to the strategy of desiccation tolerance they have evolved to use: some will have more emphasis on protection and thus less on repair and recover, others will use some constitutive protection along with an inducible component, and yet others will use variations we are yet to discover—such is the delight of working with these plants and trying to understand how they accomplish such a phenomenal feat.

2.7.1 *Constitutive Protection: Induced Repair*

The strategy of constitutive cellular protection coupled with rehydration-induced repair appears to be the first strategy to evolve for acquiring desiccation tolerance in drying habitats and is best represented (and the best studied) in the mechanism of desiccation tolerance seen in modern-day bryophytes, in particular, mosses. The constitutive aspect of the cellular protection process in bryophytes is inferred by their ability to tolerate very rapid desiccation to equilibrium and atmosphere of less than 5% RH over activated silica gel in less than 1 hour (Bewley 1979). The assumption is that desiccation, and the ensuing metabolic quiescence, occurs too quickly to initiate and establish cellular protective measures. Even under slow drying rates, to equilibrium at 67% RH within 3 to 6 hours (Oliver 1991), the amount of time available to the moss to actually redirect metabolism toward a protective posture would appear to be too short for an effective outcome. Protein synthesis is extremely sensitive to cellular dehydration and is rapidly terminated during the drying of *T. ruralis* gametophytes (Bewley 1972, 1973) and other mosses (Siebert et al. 1976, Henckel et al. 1977, Oliver et al. 1993). In *T. ruralis*, the loss of protein synthesis is manifested by a loss of polysomes that results from a runoff of ribosomes from the mRNA transcripts and a failure in the ability to reinitiate protein synthesis (see Bewley 1979, Bewley and Oliver 1992, for reviews). During slow drying, polysomes are fully depleted by the end of the initial hour of dehydration and all transcripts are free of ribosomal subunits (Oliver 1996). Rapid desiccation of *T. ruralis*, however, leads to the retention of 50% of the polysomes in the dried state, indicating that desiccation occurs so rapidly that transcripts are actually trapped within the protein synthetic machinery that itself has not had time enough to dismantle. The trapped transcripts are quickly released during rehydration and are lost from the transcript pool (Oliver and Bewley 1984b). The rapid loss of polysomes during drying and the apparent sensitivity of the initiation step of protein synthesis to protoplasmic dehydration

lead us to the conclusion that the induction of synthesis of protective proteins during drying is highly unlikely. This is borne out by the observation that no novel mRNAs are recruited into the protein synthetic complex during slow drying (Oliver 1991).

Constitutive cellular protection is also evident from the ultrastructural data that demonstrate desiccation per se does not lead to cellular damage in those desiccation-tolerant plants (again, mosses) that can dry quickly (see earlier and Tucker et al. 1975, Oliver and Bewley 1984a, Pressel et al. 2006, Proctor et al. 2007b). The need for a rehydration-induced repair process is less evident in bryophytes that have been slowly dried, although they do not recover instantaneously (Pressel et al. 2006, Proctor et al. 2007b), indicating that perhaps some damage repair is necessary. However, for rapidly dried *T. ruralis*, cellular damage is evident upon rehydration, and repair must be a necessary component of the desiccation-tolerance strategy (Oliver and Bewley 1984, aOliver et al. 1993). In addition, we do know that prolonged desiccation, especially in sunlight, results in cellular damage at all levels of organization, from proteins to chromosomes to membranes (Osborne et al. 2002), all of which has to be repaired for full recovery. Depth of desiccation (i.e., how dry they become) also affects cellular integrity as evidenced by the fact that bryophytes generally do not do well if stored below 5% to 10% RH (Proctor 2001).

There is also biochemical evidence to support a constitutive cellular protection strategy in bryophytes. Group 2 LEA proteins (dehydrins) are normally induced in response to dehydration in all plants (including desiccation-tolerant angiosperms) such that they accumulate to noticeable levels, presumably in an effort to protect cells for dehydration damage (as discussed above). In the desiccation-tolerant mosses *T. ruralis* and *Thuidium delacatulum*, these are present in the hydrated gametophytes and they do not accumulate during drying, whether drying is slow or rapid (Bewley et al. 1993). A similar trend is also true for soluble sugars, in particular, sucrose, where dehydration results in an increase in sucrose concentrations in cells of most plants, whereas in the desiccation-tolerant bryophytes, sucrose levels remain constant (Smirnoff 1992). For *T. ruralis*, the concentration of sucrose remains at 10% of the dry mass of gametophytes and does not change in amount during desiccation or rehydration in the dark or light (Bewley et al. 1978). Thus, it appears important to maintain a constant, and presumably sufficient, amount of this sugar in this moss.

All desiccation-tolerant plants that utilize constitutive cellular protection as a strategy for tolerance recover metabolic activity quickly following rehydration. The focus of that metabolic activity, whether it is for repair of damage resulting from desiccation and/or rehydration, replenishment of protective components that have been depleted during desiccation, or the reestablishment of metabolite pools for resumption of growth, remains open for debate and inquiry. The focus, however, as we have discussed will depend a great

deal on the speed at which the prior desiccation event occurred and the absolute level of dehydration the plant was subjected to; the faster the rate of drying and the drier the tissue, the more the emphasis of metabolism upon rehydration will be directed toward cellular repair. Protein synthesis, one of the first cellular processes to be affected by dehydration, rapidly recovers following rehydration. For slow-dried *T. ruralis*, protein synthesis is back to pre-desiccation levels within 2 hours following the application of water (Gwózdź et al. 1974) but closer to 3 to 4 hours if the moss is rapidly dried. Protein synthesis utilizes stored transcripts in the initial phases following rehydration, but by 2 hours the majority have been newly transcribed (Oliver and Bewley 1984c). The first 2 hours following rehydration of slow-dried *T. ruralis* mark an extensive alteration in the pattern of protein synthesis compared with that seen in hydrated gametophytes prior to desiccation (Oliver 1991). Radioactive labeling and two-dimensional gel electrophoresis revealed a decrease in the synthesis of 25 proteins (collectively termed *hydrins*) compared with predesiccation levels and an increase in synthesis of 74 proteins (collectively termed *rehydrins* [a functional term that does not indicate a common motif]) within the first 2 hours following rehydration. The synthesis of hydrins is relatively sensitive to dehydration as their synthesis declines very quickly once free water is removed. However, it takes a critical level of desiccation, between 40% and 50% of fresh weight, to initiate the synthesis of the full suite of rehydrins. With a critical water loss triggering mechanism, it appears that the moss has evolved a strategy that allows it to respond only when it is likely that the plant will desiccate rather than simply experience a short period of dehydration. This would make sense as a means of conserving energy in an uncertain environment.

A part of the strategy for plants that need to respond quickly to desiccation is that they appear to also require a rapid recovery presumably to resume growth and to make up for the time lost while dry. Part of that recovery, and a testament to the efficiency of the constitutive protection strategy, is the very rapid return to predesiccation levels of Photosystem II activity (Proctor and Pence 2002, Proctor and Tuba 2002, Proctor et al. 2007a). Obviously, the protection of the chloroplast and its energy-generating activities are paramount if growth is to rapidly reestablish following desiccation and rehydration. The need for speed is also reflected in the way that the *T. ruralis* controls the change in gene expression associated with rehydration. As will be discussed later, the common response of plants to a stressful environmental event, including dehydration, is to induce the transcription of a set of genes whose products are designed to aid the plant in either tolerating or resisting the stress. For example, drought and salt stress result in a transcriptional induction of a suite of genes, some of which encode new transcription factors, LEA proteins, aquaporins, and transport proteins (Bartels and Sunkar 2005). Such a response takes time, usually in the range of a few hours, and in these cases, plants have

evolved ways to slow the effects of the stress until such time as the processes designed to deal with them have been put in place. Bryophytes do not have that luxury and, given their environments and their lack of morphological and physiological mechanisms to slow down such events as dehydration, they have evolved ways to respond rapidly both to the stress and to recovery from it. In order to alter gene expression in such a way as to generate a rapid response, *T. ruralis* alters the pattern of protein synthesis such that rehydrins are preferentially made by directly altering the selection of transcripts destined for translation (Oliver 1991, Scott and Oliver 1994, Wood and Oliver 1999). Oliver (1991) was able to demonstrate that the transcript pool remained qualitatively constant during drying and rehydration; no entirely novel transcripts could be detected. Rehydrin transcripts accumulate to peak levels in the dried moss if desiccation is slow, apparently ready for translation once rehydration occurs (Scott and Oliver 1994). The accumulation of transcripts during slow desiccation is not the result of transcription of rehydrin genes but by the sequestration of rehydrin mRNAs in a stable form, packaged with proteins that must be preexistent in the moss (Wood and Oliver 1999). The rehydrin transcripts are packaged in the dry cells by close association with RNA binding proteins to form messenger RNA ribonucleoprotein complexes or particles, presumably protected from damage during cellular packaging and held in an untranslatable form. The assumption is that it is rehydration, perhaps a change in cellular pH or ionic status, that releases them from the sequestered form, allowing rapid recruitment into the protein synthetic complex for the biosynthesis of the various rehydrins. This is supported by their rapid inclusion in the polysomal fraction of rehydrated gametophytes (Scott and Oliver 1994, Wood and Oliver 1999). Rapid desiccation of *T. ruralis* gametophytes does not allow time for the sequestration of transcripts into mRNPs, and in this case, the moss must rely on transcripts that make it through the drying process until transcription can replenish or amass the required quantity of transcripts needed to effect recovery (Scott and Oliver 1994, Velten and Oliver 2001). This is not equivalent to a transcriptional stress response in the sense that this still occurs without a qualitative change in the transcript pool and the translational control still applies (Oliver 1991, Scott and Oliver 1994, Oliver and Wood 1997).

2.7.2 Induced Protection: Induced Repair

Desiccation-tolerant plants that require dehydration to occur slowly in order to survive desiccation generally employ a strategy of induced cellular protection coupled with, to varying degrees, a rehydration-induced repair mechanism. In the preceding narrative, I discussed the need for slow drying and how, in several desiccation-tolerant plants, dehydration results in an induction in the

synthesis and accumulation of both LEA proteins and sugars, major components of cellular protection strategies. In the case of *C. plantagineum*, the accumulation of sucrose in response to desiccation is the result of an induction of the conversion of stored 2-octulose to sucrose rather than its de novo synthesis or from the breakdown of starch reserves (Bianchi et al. 1991). However, it is in the control of gene expression in response to dehydration that we really uncover the intricacies of the induction of cellular protection processes that mark this strategy for desiccation tolerance.

Signal-transduction leading to the induction of gene expression in response to dehydration (not desiccation) in angiosperms has been extensively studied in an effort to understand and to develop drought-tolerant crops (Shinozaki and Shinozake 1997, Ramanjulu and Bartels 2002, Zhu 2002, Bartels and Sunkar 2005). It is clear that gene expression responses to water deficit stress are complex; over 4391 genes are upregulated by drought in *Arabidopsis* (Rizhsky et al. 2004), and the signal-transduction pathways that control the response are equally intricate. It is clear, however, that the environmental cues that are translated into a gene expression response are delivered via a network of signals that include both ABA-directed and non-ABA-directed pathways (Shinozaki and Shinozake 1997, Ramanjulu and Bartels 2002, Zhu 2002, Bartels and Sunkar 2005). ABA has long been known to play a critical role in the acquisition of desiccation tolerance in orthodox seeds (Bewley and Black 1994), and so it is not surprising that ABA has been demonstrated to play a major role in the acquisition of desiccation tolerance in vegetative tissues (Phillips et al. 2002). In *C. plantagineum* leaf tissues, ABA increases 6- to 7-fold during slow drying (Bartels et al. 1990), as seen in other desiccation-tolerant angiosperms (Gaff and Loveys 1984). During drying of *C. plantagineum*, many new proteins are synthesized in leaf tissues at the time of the observed increase in ABA content, and in addition, many appear when ABA is applied to nonstressed tissues such as callus, which acquire desiccation tolerance in response to ABA (Bartels et al. 1990). Differential screening of cDNA libraries enabled the isolation of cDNAs representing transcripts expressed only in desiccation-tolerant tissues of *C. plantagineum* (Bartels et al. 1990, 1993) that have since been effectively used to unravel some of the nuances of the induction of cellular protection processes. Analysis of the promoter regions of several of the genes that correspond to the transcripts that are induced by both ABA and desiccation has not revealed a common motif that could be ascribed as possible common ABA- or desiccation-regulated transcription factor binding site (Michel et al. 1993, 1994, Velasco et al. 1998). The implication from these studies is that there are multiple pathways for ABA signal transduction in *C. plantagineum*.

The idea that *C. plantagineum* and, by inference, other resurrection species, use novel signal-transduction pathways to induce cellular protection strategies for desiccation tolerance appears to be born out by the following studies. The

existence of a novel DNA-binding protein (or proteins) that binds to the promoter for one of these genes (*pcC27-45*) in a sequence-specific manner and is also ABA responsive has been demonstrated (Nelson et al. 1994). The sequences to which this factor (or these factors) binds appear to be related to sequences present in seed storage protein promoters (which have not been determined to be ABA regulated). Promoters, from the genes *pcC6-19* and *pcC27-45*, were analyzed in transgenic tobacco and *Arabidopsis*, and both promoters were highly active in tissues that experience desiccation (i.e., seeds and pollen). However, only the promoter from *pcC6-19* was induced by dehydration or ABA in the vegetative tissues of the transgenic plants (Furini et al. 1996), and ectopic expression of the ABI-3 gene product rescued the ABA-inducible activity of the *pcC27-45* promoter, implying that the activity of transcription factors present in the leaves of *Craterostigma* are absent in tobacco and *Arabidopsis*. Using the yeast one-hybrid screen to identify cDNAs encoding DNA-binding proteins that attach to the promoter of *pcC27-45*, Hilbricht et al. (2002) were able to identify a novel SAP-domain plant transcription factor (CpR18) that binds to the domain of the promoter necessary for ABA- and dehydration-induced expression. Other studies have identified other transcription factor families involved in gene regulation in response to desiccation in *C. plantagineum* including members of the *myb* transcription factor family (Iturriaga et al. 1996), a heat shock transcription factor (Bockel et al. 1998), and members of the leucine zipper family (Frank et al. 1998).

That the signal-transduction pathways for desiccation tolerance are different from those normally associated with dehydration responses in angiosperms is most distinctly seen in the discovery, using tDNA activation tagging, of a family of novel signaling molecules in the ABA transduction pathway for *C. plantagineum*, coded for by a small gene family termed *Craterostigma* desiccation-tolerant (*CDT*) genes (Furini et al. 1997, Smith-Espinoza et al. 2005). *CDT-1*, the first gene of this family to be identified, was shown to have structural features that resemble a group of SINE-retrotransposons, suggesting that these genes function by expressing a small regulatory RNA molecule rather than a protein (Furini et al. 1997). Constitutive overexpression of *CDT-1* in transgenic callus from *C. plantagineum* conferred desiccation tolerance on the tissue even in the absence of ABA and in addition resulted in the constitutive expression of the proteins characteristic of ABA- and dehydration-induced gene expression. *CDT-2* acts in ways similar to *CDT-1* in that both activate a suite of *Lea* genes in callus tissues; however, *CDT-2* affects the expression of a several *Lea* genes in leaves that *CDT-1* does not (Smith-Espinoza et al. 2005). A further *CDT* gene, *Cdt-3*, does not appear to activate the *Lea* genes that *Cdt-1/2* control but does affect internode length as seen in *cdt-3* mutant plants. The phenotype of *cdt-3* mutants, callus desiccation tolerance and altered internode length, infers a connection between ABA and Gibberellin signal-transduction pathways (Smith-Espinoza et al. 2005). The absence of

enhanced levels of ABA-dependent transcripts in the *cdt-3* mutant suggests that desiccation tolerance in *C. plantagineum* may not always involve ABA-dependent pathways.

The inference that the ABA signaling pathway is not the sole mechanism by which cellular protection is induced in desiccation-tolerant plants had first been proposed by Gaff and co-workers working with the desiccation-tolerant grass *S. stapfianus*. In this plant, only leaves that are attached to the parent plant survive desiccation; detached leaves die (Gaff and Ellis 1974). Application of exogenous ABA does not allow detached leaves to survive desiccation (Gaff and Loveys 1993). However, if the parent plant is allowed to dry to 61% relative water content or lower, leaves can be removed and they will tolerate desiccation (Kuang et al. 1995). At this level of dehydration, endogenous ABA levels have only just started to rise and the peak of ABA accumulation is much later. In addition, the synthesis of many of the desiccation specific proteins is induced at relative water contents much higher than 61% (Kuang et al. 1995). These types of observations tend to suggest that the ABA signal transduction pathway is not a major player in the induction of cellular protection processes in *S. stapfianus*. The involvement of non-ABA pathways of signal transduction in the establishment of desiccation tolerance was confirmed by the discovery that dehydration caused the activation of phospholipase D activity in *C. plantagineum* (Frank et al. 2000). Phospholipase D, an important component of early signaling cascades in both plants and animals, is activated within minutes of the onset of dehydration. Its activity was not inducible by ABA but did respond to the application of mastoparan, a G protein-activating compound, indicating that the induction of phospholipase D activity is part of the activation of a signaling pathway. It is possible that this pathways as well as others not yet identified that operate in the induction of desiccation tolerance and, by inference, the cellular protection processes in *S. stapfianus*.

Obviously, there must be a great deal of further inquiry into these pathways and the transcription factors that are involved before we can fully understand what is required for the induction of cellular protection in desiccation-tolerant plants.

The need for repair following rehydration of plants that use the induced cellular protection strategy has been much debated, but as I have discussed previously, damage does occur and, as in all species, it is the speed of drying, time spent dry, and the depth of desiccation that control the extent to which it occurs. For example, rapid-dried *C. wilmsii* is fully capable of limiting damage to a point where it can survive such a treatment; however, if RNA or protein synthesis is inhibited during the rehydration phase, damage is not limited and the plant cannot recover (Cooper and Farrant 2002). Clearly, this demonstrates the need for an inducible repair system that is activated during rehydration. In contrast, rehydration of *C. plantagineum* revealed little in the way

of induced gene expression; only a very few transcripts were identified as being specific for the rehydration process (Bernacchia et al. 1996). Those that were appeared to be transketolases involved in the metabolism of sugars required to reestablish the pools of octulose required for the generation of sucrose during dehydration (Bernacchia et al. 1995). Studies aimed at isolating transcripts that are specific to rehydration of slow-dried *S. stapfianus* resulted in the isolation of only two genes, both of which are induced in the early stages following the initial addition of water to the plants but both are also induced during drying. One of the transcripts encodes a plant Rab2, a small GTP-binding protein that in other systems is an important protein in the targeting of membrane vesicles in vesicular trafficking pathways and a pathway directly involved in membrane construction (O'Mahony and Oliver 1999a). The other encodes a polyubiquitin protein that is involved in the turnover of damaged proteins (O'Mahony and Oliver 1999b). All of these studies have used plant materials that have been exposed to ideal and relatively innocuous drying regimens, and so one could expect little in the way of desiccation-induced damage or a rehydration-induced repair process. It would be much more interesting to look at field dried material for such a repair process and the genes involved in it.

2.8 Genomics and Desiccation Tolerance

With the exception of the relatively short list of genes or gene products (e.g., LEAs, HSPs, and enzymes involved in sucrose metabolism), we know very little about what the effect of desiccation and rehydration has on the transcriptome, the proteome, or the metabolome of desiccation-tolerant plants. We are a long way behind what is known about the transcriptome-level response to drought in sensitive species, for instance (Rizhsky et al. 2004). Nevertheless, genomic level studies have been initiated, most focused on the transcriptome and expression profiling, to discover the extent of the effect of desiccation and/or rehydration, how gene expression is controlled, what processes are affected, the discovery of genes novel to the study of dehydration and desiccation tolerance, and, last but not least, the discovery of those genes that actually control the induction and acquisition of desiccation tolerance in plants.

In an effort to understand what processes were involved in the repair and recovery of desiccation-tolerant bryophytes upon recovery, a collection of expressed sequence tags (ESTs) was started for the bryophyte model plant *T. ruralis* (Wood and Oliver 2004). The rationale behind this endeavor was to create a genomic resource that would not only provide the tools necessary to explore the rehydration transcriptome but also provide the basis for future genome mapping projects. The first 18 cDNAs that initiated this collection

were obtained from the differential screening of a gametophytic rehydration cDNA library (Scott and Oliver 1994); these were later joined by the isolation of 152 EST clones derived from a library constructed from RNA sequestered in the mRNP fraction of slow-dried gametophytes (Wood et al. 1999). Of these 152 ESTs, only 29% exhibited any sequence similarity to previously identified sequences deposited in public databases. Of those ESTs that could be annotated, several were identified as encoding ribosomal proteins, which is not surprising given the importance of translation in the response of this moss to desiccation and rehydration. Also of interest was the identification of several LEA protein transcripts and an early light inducible protein (ELIP) that is known to be a chloroplast protection protein. The latest addition to the *T. ruralis* desiccation/rehydration EST collection was a relatively large one of 10,368 ESTs from a non-normalized rehydration specific library (Oliver et al. 2004). The 10,368 ESTs represent 5,563 clusters (contig groups representing individual genes), of which 40.3% could not be assigned an identity by comparison to annotated sequences in the public databases (Oliver et al. 2004). A qualitative look at transcript abundance during the recovery phase by Genome Ontology (GO) mapping of the *Tortula* clusters gave a broad look at what cellular activities appear to be emphasized in the rehydrated gametophytes. This analysis confirmed that the protein synthetic machinery, membrane structure and metabolism, and plastid integrity are all central to the response. The GO analysis also revealed previously nonresearched areas of cellular recovery such as membrane transport processes, phosphorylation, and signal transduction (Oliver et al. 2004). Signal transduction is especially intriguing given that the change in gene expression in these plants in response to desiccation is at the translational control level.

Of the 30 most abundant transcripts, by virtue of the number of ESTs representing a particular transcript, present in the early phases of recovery following rehydration, 7 encode LEA or LEA-like proteins (Oliver et al. 2004, 2005), including one that we consider to be a “primitive” dehydrin like LEA, Tr288 (Velten and Oliver 2001). The emphasis on LEA transcripts in the initial phases of rehydration is somewhat surprising; it may indicate a need to replenish the pool of LEA proteins that were used or lost during desiccation. Some LEA proteins may play a dual role by protecting cells during both dehydration and rehydration. A more unconventional possibility is that LEAs in the moss are there to provide protection from the rigors of rehydration and are not active during desiccation. Could this actually be the role of LEA proteins in all desiccating tissues? This is a hypothesis worth testing in the future. The significance of the crude bioinformatics measure of transcript abundance was elevated by an expression profile study using a cDNA microarray constructed from the 5563 individual clusters derived from the large EST collection (Oliver et al. 2005). Twenty-four of the clusters that exhibited at least a 2-fold increase in transcripts accumulation levels within gametophytes that had been

rehydrated for between 1 and 2 hours have sequence similarity to known LEA protein sequences. These transcripts are also elevated greater than 2-fold in the polysomal RNA fraction, indicating their recruitment into the translational mRNA pool. Because each cluster represents a unique nucleotide sequence, it would suggest that following rehydration, *T. ruralis* has a wide range of LEA-like proteins available either for replenishment of the LEA protein pools that are constitutive or for use in the recovery process.

Genomic level studies in desiccation-tolerant tracheophytes are also under way. Early studies for *C. plantagineum* used differential, subtractive, or cold plaque screening to isolate and characterize 200 cDNA clones from leaves dried for 1 hour or to complete dryness (Bockel et al. 1998) in order to isolate genes associated with the early stages of the acquisition of tolerance (as discussed in the previous section). Genes encoding abundant drought-induced genes correlated with desiccation tolerance or low abundance transcripts encoding gene products not previously associated with drought stress in *S. stapfianus* were isolated by differential screening (Blomstedt et al. 1998) or by cold-plaque hybridization procedures (Neale et al. 2000), respectively, suggesting that *S. stapfianus* may possess unique genes and/or regulatory processes that confer desiccation tolerance.

Recently, 1046 ESTs from a cDNA library constructed from whole plants of the desiccation-tolerant spikemoss *Selaginella lepidophylla* undergoing desiccation were isolated and characterized (Iturriaga et al. 2006). These 1046 ESTs represent 873 unique transcripts. Prior to this, 2181 ESTs representing 1301 putative unigenes were collected from a nontolerant sister species, *Selaginella moellendorffii* (Weng et al. 2005). Comparison of the *S. lepidophylla* ESTs with the 1301 unigenes from *S. moellendorffii* revealed that 63% of genes were unique to *S. lepidophylla*. In contrast to *S. moellendorffii*, the desiccation-tolerant *S. lepidophylla* EST collection contained a much greater relative percentage of stress-response (i.e., LEA protein), chaperone, and HSP ESTs. More important, analysis of the most abundant transcripts revealed that *S. lepidophylla* preferentially expressed genes whose primary assignable function is in stress-response pathways (Iturriaga et al. 2006). The value of comparative genomics, especially comparison of sister species that differ primarily in one trait—in this case, tolerance to desiccation—cannot be understated. It is by this method that we will begin to fully understand which genes involved in desiccation and rehydration responses are truly adaptive and hence central to desiccation tolerance.

The power of comparative genomics has also been demonstrated recently with a small genomics effort centered on the desiccation response of the desiccation-tolerant plant *X. humilis* (Collet et al. 2004, Illing et al. 2005). From a normalized cDNA library generated from RNA isolated from leaves and roots at various stages of drying and rehydration, Collett et al. (2004) isolated and rapid-sequenced 429 cDNA ESTs. These ESTs were arrayed and used in a small

EST profiling study, to reveal 49 ESTs that were induced as a consequence of dehydration. Within these dehydration-induced transcripts were several with homology to drought-responsive genes as well as novel genes, but the overwhelming majority (16) encoded LEA proteins including dehydrins. As part of an exciting comparative study, Illing et al. (2005) compared the expression of the dehydration-induced transcripts with publicly available expression data for developing seeds of *Arabidopsis* and found that the *Arabidopsis* counterparts for two of the dehydration upregulated genes in the leaf tissues of *X. humilis*, a LEA-6 and a 1-cys-peroxiredoxin gene, were seed specific in their pattern of expression. This observation led Illing et al. (2005) to suggest that the mechanism for vegetative desiccation tolerance in these plants carries “footprints” of a seed-derived origin as suggested by the phylogenetic analyses of Oliver et al. (2000, 2005) and is the first experimental evidence for this hypothesis.

Bioinformatics comparison of *T. ruralis* transcript expression profile data (Oliver and Payton, unpublished data), using cDNA arrays developed from a unicluster EST set derived from Oliver et al. (2005) and oligoarray data generated by Rizhsky et al. (2004) for gene expression profiles in *Arabidopsis* responding to drought stress have revealed extensive differences in how these two organisms respond to dehydration stress. Using the *Arabidopsis* uniprot-protein sequence database (www.arabidopsis.org), 775 of the 5,563 *Tortula* EST clusters could be assigned an *Arabidopsis* counterpart in the genes that exhibit an alteration in expression in response to drought stress in *Arabidopsis* in the Rizhsky et al. study (2004). As depicted in Fig. 2.2, within the 4,391 genes

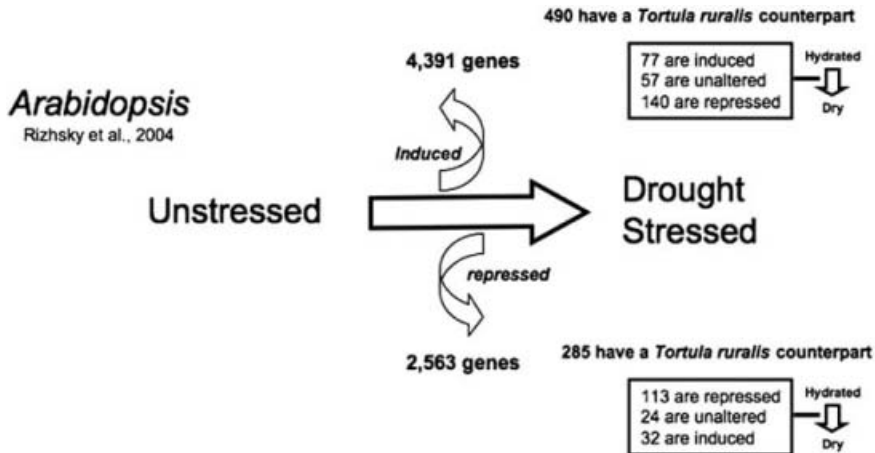


Fig. 2.2 Diagrammatic representation of the bioinformatics and expression profile comparison of the genes that respond to drought conditions in *Arabidopsis* (derived from Rizhsky et al. 2004) with the response of *Tortula ruralis* presumed homologs during desiccation (from Oliver and Payton, unpublished data).

that are induced by drought in *Arabidopsis*, 490 have a *Tortula* counterpart; however, within that 490, only 77 are induced, and of the others, 57 are unaltered and 140 exhibit repression in *Tortula*. The remainder did not exhibit significant alterations in expression in *Tortula*. Within the 2,563 genes that are repressed by drought stress in *Arabidopsis*, 285 had a *Tortula* counterpart; however, only 113 of these are repressed, 24 are unaltered, and 32 exhibit induction in *Tortula*. This analysis reveals a fundamental difference in how *Arabidopsis* genes respond, compared with the response of their “homologs” in *T. ruralis*, to dehydration, indicating that perhaps the water deficit response of desiccation-sensitive plants has evolved from a different origin or via a very different pathway than the dehydration response in desiccation-tolerant plants.

2.9 Concluding Remarks

In this chapter, I have tried to provide a solid overview of what we have learned about how extremophiles cope with the desiccation of their vegetative tissues that they regularly experience in the habitats for which they evolved. I have also tried to introduce the underlying hypotheses that drive inquiry into this trait as well as several of the main areas of interest in this field. As we move into the “omics” era, we have a strong biological understanding of much of how plants tolerate desiccation, and the insights that genomic, proteomics, metabolomics, and other “omic” level studies will provide will be well served by this solid knowledge base. It is an exciting time to be involved in the field of desiccation tolerance in plants, and much will be learned in the very near future. This bodes well for the expressed desire of most who work in this field, to discover new genes and new strategies for crop improvement designed to deliver more drought-tolerant plants.

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3 Mechanisms of Desiccation Tolerance in Angiosperm Resurrection Plants

Jill M. Farrant

3.1 Introduction: Some Definitions and Preliminary Explanations

In this chapter, the ability of vegetative tissues of a few angiosperm species, termed *resurrection plants* (Gaff 1971), to tolerate loss of more than 95% of their cellular water is reviewed. The stresses imposed by water loss and the suspected mechanisms whereby they achieve survival of this loss will be reviewed from the whole plant to the cellular levels. In the lower orders (bryophytes and lichens), desiccation occurs very rapidly, and protection prior to drying is minimal and constitutive. Survival is thought to be based largely on rehydration-induced repair processes (Oliver and Bewley 1997, Oliver et al. 1998, Alpert and Oliver 2002). In angiosperm vegetative tissues, while some repair is probably inevitable, considerable and complex protection mechanisms are laid down during drying to minimize the need for extensive repair (Gaff 1989, Farrant 2000, Scott 2000, Alpert and Oliver 2002, Viret et al. 2003, 2004a, Bartels 2005, Illing et al. 2005). This review concentrates on some of the protection mechanisms displayed by selected angiosperm species (see Table 3.1, bold type, and Figs. 3.1, 3.2, and 3.5). For comparison, where applicable, the responses of selected desiccation-sensitive (DS) species are reviewed. For example, in the genus *Eragrostis*, there are species with differing degrees of tolerance to water deficit that serve as a good comparative model system. *E. nindensis* (Fig. 3.5A,B) is the only resurrection species tolerating drying to 5% relative water contents (RWC), but *E. curvula*, *E. teff*, and *E. capensis* have critical water contents below which they cannot be dried of 45%, 50%, and 65%, respectively (Balsamo et al. 2005, 2006).

Desiccation tolerance (DT) in the vegetative tissues in angiosperms is rare, with only some 300 species being reported to tolerate such drying, these occurring predominantly in the southern hemisphere in Africa, America, and Australia (Gaff 1977, Alpert and Oliver 2002). While there are no real phylogenetic relationships with respect to vegetative DT in the angiosperms, among the dicotyledonous plants there are several members in Schrophulariaceae (32 species) and Myrothamnaceae (2 species), and in the monocots, there are several representatives of the Poaceae (36 species) and Xerophytaceae (28 species) (Gaff 1971, 1977, Proctor and Tuba 2002) (Table 3.1). The more unifying feature of these plants is their occurrence mainly in shallow soils on rocky outcrops in semitropical and tropical regions (Porembski and Barthlott 2000). Under these conditions

Table 3.1 Angiosperm resurrection plants for which there is reported data on mechanisms of desiccation tolerance.

Family, Genus & Species	Growth Form and Some Characteristics Important to DT	Longevity in the Dry State (Month)	Some Important References
DICOTYLEDONS			
Schrophulariaceae (32)			
<i>Craterostigma plantagineum</i>	In all <i>Craterostigma</i> spp. rosette growth form; outer leaves curl and shade inner leaves when dry. All are H	nd	Bartels et al., 1990; Bianci et al., Bernacia et al., 1996; Ingram & Bartels, 2000; 2003; Jones & McQueen-Mason; 2004; Hoekstra, Sherwin and Farrant, 1996; 1998; Cooper and F Farrant et al., 1999, 2003; Farrant, 2004a,b
<i>C. pumilum</i>	WF	11–24; lab	
<i>C. purpureus</i>	nd	24; lab	
<i>C. wilmsii</i>	WF	3; field	
(Fig. 3.1 A,B)			
<i>Lindernia</i> spp (> 15)	nd	nd	Rahmanzadeh et al., 2005; Phillips et al.,
Myrothamnaceae (2)			
<i>Myrothamnus flabellifolius</i>	Shrub, max height 1.5 m; Leaves fold fan-like and upwards when dry. H;	6–12; field 84; lab	Gaff, 1977; Sherwin and Farrant, Farrant & Kruger, 2001; Farrant, Kramer & Birtic, 2005; Moore et al., Walters et al., 2005
(Fig. 3.1 C,D)	WF+VF		
Gesneriaceae (2)			
<i>Boea hygroscopea</i>	Rosette growth form; H	nd	Bohicchio et al., 1998
<i>B. hygrometrica</i>	Rosette growth form; H	nd	Deng et al., 2003
Liliaceae (3)			
<i>Borja nitida</i>	Small, shrub-like; P	nd	Hetherington and Smillie, 1982
<i>Chamaejasme</i>	Suspended in water pools in rock indentations	11, field	Hartung et al., 1998; Schiller et al.,
interpidis			

MONOCOTYLDONS

Velloziaceae (157)					
Xerophytaceae					
<i>Xerophyta humilis</i> (Fig. 3.2 C,D)	± 5 cm height, grows in mats; P; VF	>10 m; field			Dace et al., 1998; Farrant, 2000; F et al., 2003; 2004; Illing et al., Gaff, 1977
<i>X. retinervis</i>	± 1 m in height, individual growth; P; VF	nd 24; lab			Hallum and Luff, 1980
<i>X. villosa</i>	± 0.5 m, individual growth, P; nd	nd			Tuba et al., 1993a,b; 1994; 1998
<i>X. scabrida</i>	± 0.5 m, individual growth, P; nd	nd			Sherwin and Farrant, 1996; 1998; Mundree & F
<i>X. viscosa</i> (Fig. 3.2 A,B)	± 0.5 m, individual growth, P; VF	nd			Mundree et al., 2000; 2002; Ndima et al., 2002; Ekmecki et al., 2004; Marais et al.,
Poaceae (36)					
<i>Eragrostis nindensis</i> (Fig. 3.3 A,B)	15–20 cm, tussock P; VF in bundle sheath cells; WF in mesophyll cells	nd			Vander Willigen et al., 2001; 2003,
<i>Sporobolus stapfianus</i> (Fig. 3.3 C,D)	20–30 cm tussock P; VF in bundle sheath cells; WF in mesophyll cells	nd			Blomstedt et al., 1998; Ghasempour et al., Whittaker et al., 2001; 2004; Neale et al.,

The numbers of DT members in the respective families and genera are given in parentheses in the first column. Some whole plant tial role in survival are given in column 2. These include retention of chlorophyll (homoiochlorophyll, H) or loss of chlorophyll anisms to avoid free radical (ROS) formation. Wall folding (WF) or vacuole filling (VF) or both (WF+VF) are mechanisms to a in the dry state, where known, is given in months in column 3. nd = not determined.

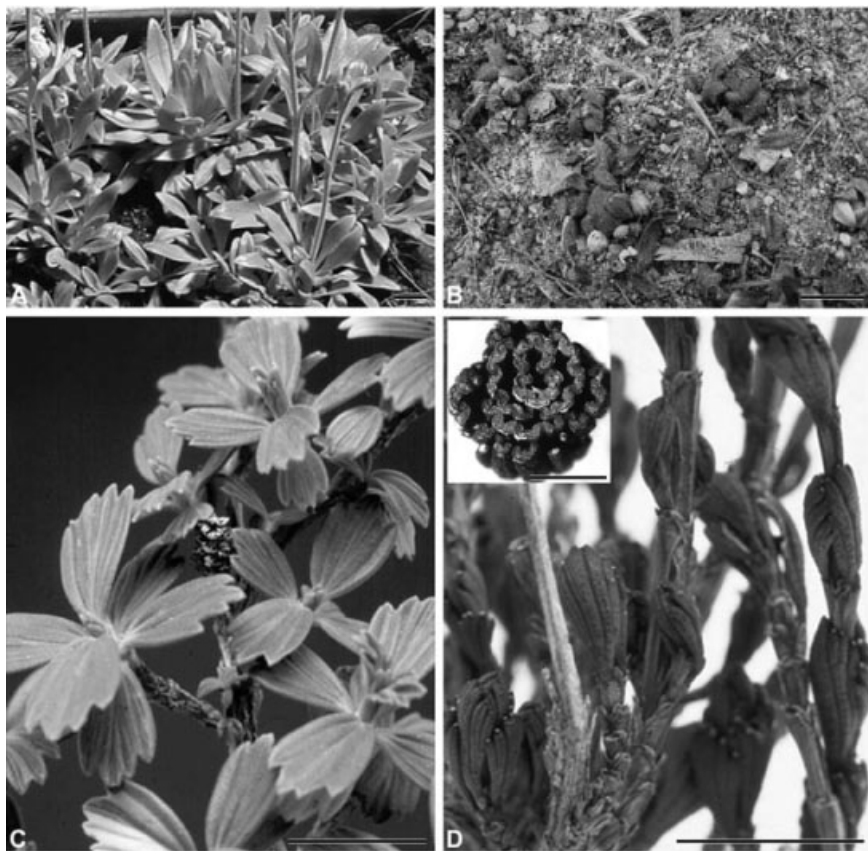


Fig. 3.1 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) dicotyledonous resurrection plants *Craterostigma wilmsii* (A,B) and *Myrothamnus flabellifolius* (C,D). Inset, dry leaves of *M. flabellifolius*, showing leaf curling and retention of chlorophyll in the shaded (adaxial) surfaces and waxy anthocyanin containing outer (abaxial) surfaces. For color detail, please see color plate section.

they are subject to frequent cycles of drying and rehydration throughout the year and thus tolerate being dry under both hot and cold environmental conditions. However, under natural conditions, the majority of time in the dry state is usually spent in the nonrainy season, which in the southern African species reported on here is winter. We have shown (Table 3.1, J. Bajic and J. M. Farrant, unpublished data) that there is greater longevity in the dry state under cold conditions, although this might be a function of slower biochemical deterioration under these conditions. Nevertheless, many of the angiosperm resurrection plant species, such as *Xerophyta viscosa* and *Myrothamnus flabellifolius*, are true extremophiles, tolerating not only desiccation but also flooding and thus short-term root anoxia due to the rocky substrates on which they grow (Figs. 3.1 and 3.2). They

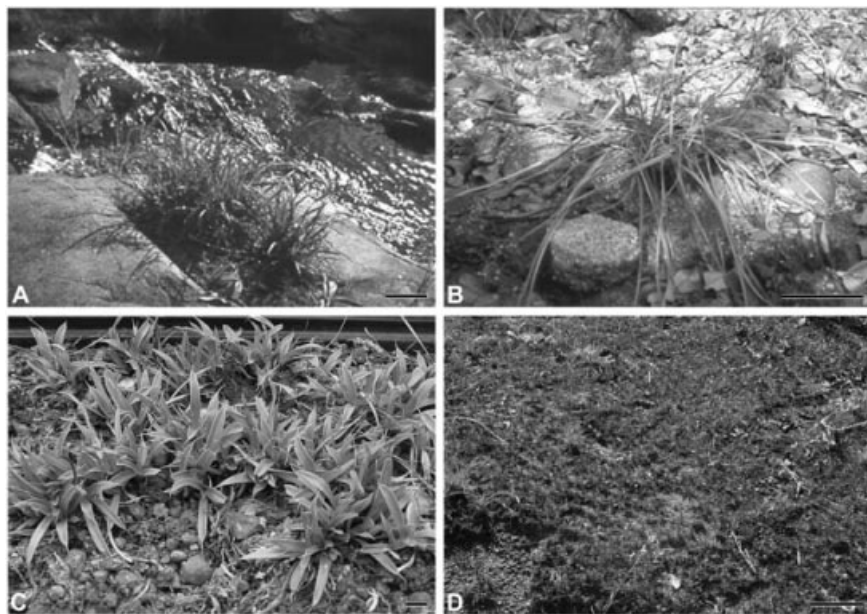


Fig. 3.2 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) monocotyledonous resurrection plants *Xerophyta viscosa* (A,B) and *Xerophyta humilis* (C,D). For color detail, please see color plate section.

are also tolerant of temperatures ranging from -4°C to 60°C (sometimes in a day) (Mundree et al. 2002, Moore et al. 2005a, 2007a).

Albeit that in a book such as this there should be no need to define the term “desiccation tolerance,” it is important to point out some features of DT that are pertinent to vegetative tissues. The definitions, terminology, and much of the understanding of anhydrobiosis have come from work done on desiccation-tolerant (orthodox) seeds. Thus, the definition of DT is usually cited as “the ability to survive drying to, or below, the absolute water content of $0.1 \text{ g H}_2\text{O} \cdot \text{g}^{-1}$ dry mass ($\text{g} \cdot \text{g}^{-1}$), this being equivalent to air-dryness at 50% relative humidity and 20°C and corresponding to a water potential of $\leq -100 \text{ MPa}$ (Vertucci and Farrant 1995, Walters et al. 2005, Berjak 2006). While the vegetative tissues of the angiosperm plants discussed here undoubtedly dry to equilibrium of the surrounding air and do reach water contents of $\leq 0.1 \text{ g} \cdot \text{g}^{-1}$, experiments are usually performed under glass house or simulated field conditions (as opposed to maintenance under constant relative humidity (RH) and temperatures typical in seed storage experimentation) and the RH and temperature can vary within and among the various experimental designs. Under such conditions, slight but immeasurable fluctuations in tissue water content may occur even when plants are supposedly being maintained

in the dry state. It has been argued that in air-dried seeds, internal water distribution may not be equal and localized pockets of water might enable preferential hydration of important macromolecules, cells, and tissues (Leubner-Metzber 2005, Oracz et al. 2007). I believe this is even more likely to be true in the air-dry vegetative tissues of resurrection plants (elaborated on later). Indeed, we have demonstrated the presence of “mobile water” in air-dry leaves of *E. nindensis* (Balsamo et al. 2005).

Another factor pertinent to studies on whole plants is that water contents at full turgor can vary extensively among species, and thus in order to compare critical water contents among species, it has become the habit of researchers in this field to use RWC. This is the water content of a tissue relative to the full turgor condition and is calculated as the absolute water content, determined gravimetrically by oven drying at 70°C for 48 hours, divided by the gravimetric water content determined at full turgor. Full turgor water content is determined from well-watered plants that have been sealed overnight in plastic bags. I will be using this terminology in this review but, where possible, will convert to absolute water content in $\text{g} \cdot \text{g}^{-1}$. Figure 3.3 shows the drying curves of the leaves of resurrection plants reported on here. The smaller species (*C. wilmsii*, *X. humilis*, and *E. nindensis*) dry more rapidly than do the larger ones (*X. viscosa* and *M. flabellifolius*). What appears to be characteristic of all these species is that leaf RWC remains high and at near full turgor for some time, essentially tracking soil water content (not shown, but reported;

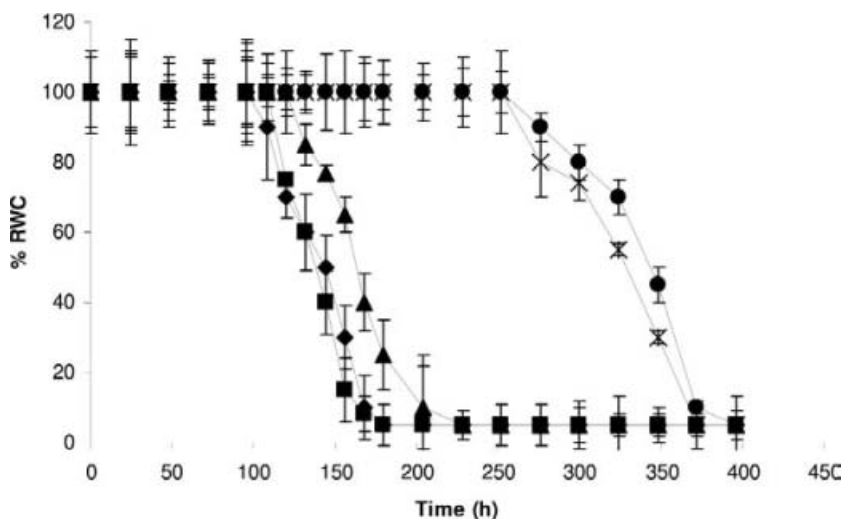


Fig. 3.3 Drying time curves for *C. wilmsii* (closed diamonds); *M. flabellifolius* (closed circles); *X. humilis* (closed squares); *Eragrostis nindensis* (closed triangles); *X. viscosa* (stars). Data taken from Farrant et al., 1999; Vander Willigen et al., 2001 and Mowla et al., 2002.

e.g., Sherwin and Farrant 1996), but when water begins to be lost from the leaves, it is extremely rapid, in the order of 1 to 2 days. During this period of rapid drying, stomata open and water appears to be actively lost from the tissues (Vicre et al. 2004a, Moore et al. 2007a, 2007b) (Fig. 3.4).

3.1.1 Stresses Associated With Drying—and the Solutions Offered

Water plays many and varied roles in plant tissues. It fills intracellular spaces, providing structural support or *mechanical stabilization* (Levitt 1980). It is involved in metabolism as both a reactant and a product of many processes, and it is the medium in which the intracellular milieu is suspended. By providing hydrophobic and hydrophilic interactions, it determines conformation of macromolecules and membranes and controls and maintains intracellular distances among them (*metabolic stabilization*) (Vertucci and Farrant 1995, Hoekstra et al. 2001, Buitink et al. 2002, Walters et al. 2002). Details of the consequences of loss of this water are considered in depth elsewhere in this book, but in reviewing the mechanisms of protection apparently adopted by angiosperm resurrection plants as a consequence of desiccation, it is important to briefly outline some of the main stresses associated with extreme loss of water.

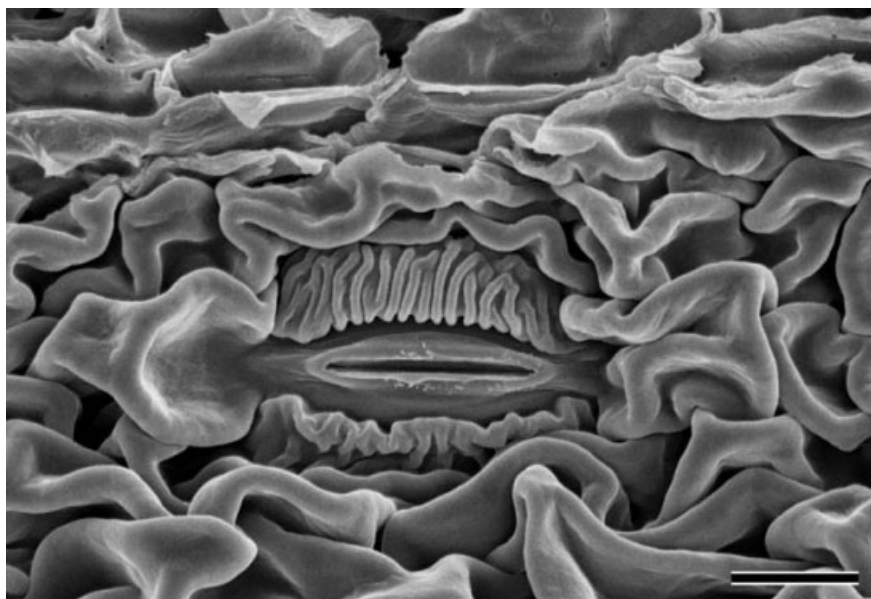


Fig. 3.4 Scanning electron micrograph of open stomata in leaves of *X. viscosa* at RWC of 25%. Material preparation and SEM as described in Figs. 3.6 and 3.7. Scale bar = 10 μ m.

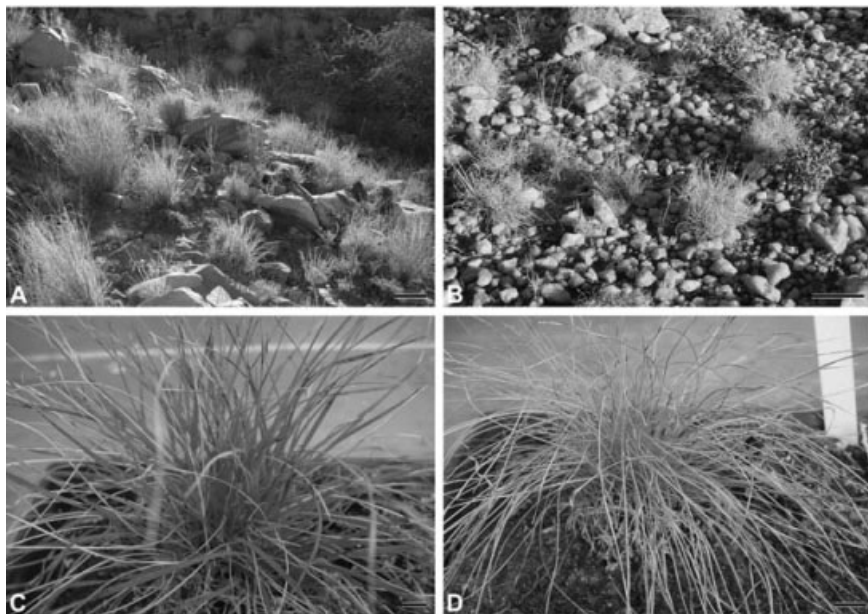


Fig. 3.5 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) grasses *Eragrostis nindensis* (A,B) and *Sporobolus stapfianus* (C,D). For color detail, please see color plate section.

3.1.2 Mechanical Stress

Mechanical stress occurs due to the loss of turgor and cell volume as water is lost and was proposed by Iljin in 1957 to be one of the major causes of irreversible desiccation-induced damage in plants. At the cellular level, loss of water from vacuoles and cytoplasm causes tension on the plasmalemma as it shrinks from plasmadesmatal attachments to the cell wall. Increasing compaction of organelles and macromolecules and ultimate rupture of the plasmalemma, allowing entry of extracellular hydrolases, result in lethal damage and cell death (Walters et al. 2002).

Leaf and root tissues of angiosperm resurrection plants undoubtedly undergo considerable shrinkage (Figs. 3.6 and 3.7) and morphological change during drying (Figs. 3.1, 3.2, and 3.5 through 3.7), with the degree of shrinkage being greater in the dicots, where wall folding plays an important role in mechanical stabilization. They are able to survive these changes by active induction of protection mechanisms that allow avoidance of plasmalemma rupture and wall collapse.

There appears to be two general mechanisms used by angiosperm resurrection plants to avoid mechanical stress: active and reversible wall folding as seen in the *Craterostigma* spp. (Vicre et al. 1999, 2003, 2004b) (Fig. 3.8A)

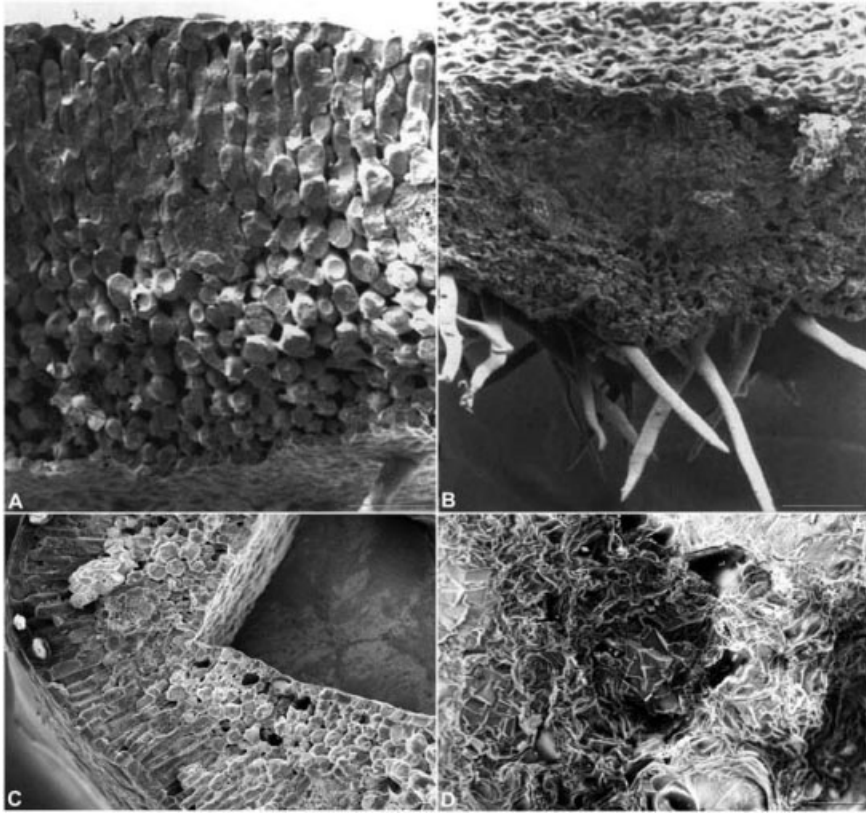


Fig. 3.6 Scanning electron microscopical images of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) leaves of the dicots *Craterostigma wilmsii* (A,B) and *Myrothamnus flabellifolius* (C,D). Scanning electron microscopy was performed using a Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo Transfer System. Leaves from hydrated and desiccated plants were frozen using liquid nitrogen and viewed directly or after freeze-fracturing. Scale bar in A,B = 50 μm ; C,D = 200 μm .

and increased vacuolation with water replacement in vacuoles by nonaqueous substances such as in the *Xerophyta* spp. (Farrant 2000, Mundree and Farrant 2000) (Figs. 3.9A, B). Some species, such as *M. flabellifolius* (Fig. 3.9C), *E. nindensis* (Figs. 3.8B and 3.9D), and *S. stapfianus* (Figs. 3.10 and 3.14), use both mechanisms, usually in different tissues. In the grasses, wall folding occurs in the mesophyll and vacuole filling in the bundle sheath cells (vander Willigen et al. 2003, 2004). DS species show neither mechanism, and subcellular damage is lethal, as is illustrated in Figure 3.10 for *E. curvula*. Interestingly, while resurrection plants adopt one (or both) of these general strategies, the manner in which they achieve it varies among the species (explained later). I propose that this reflects multiple evolutions of the same strategy.

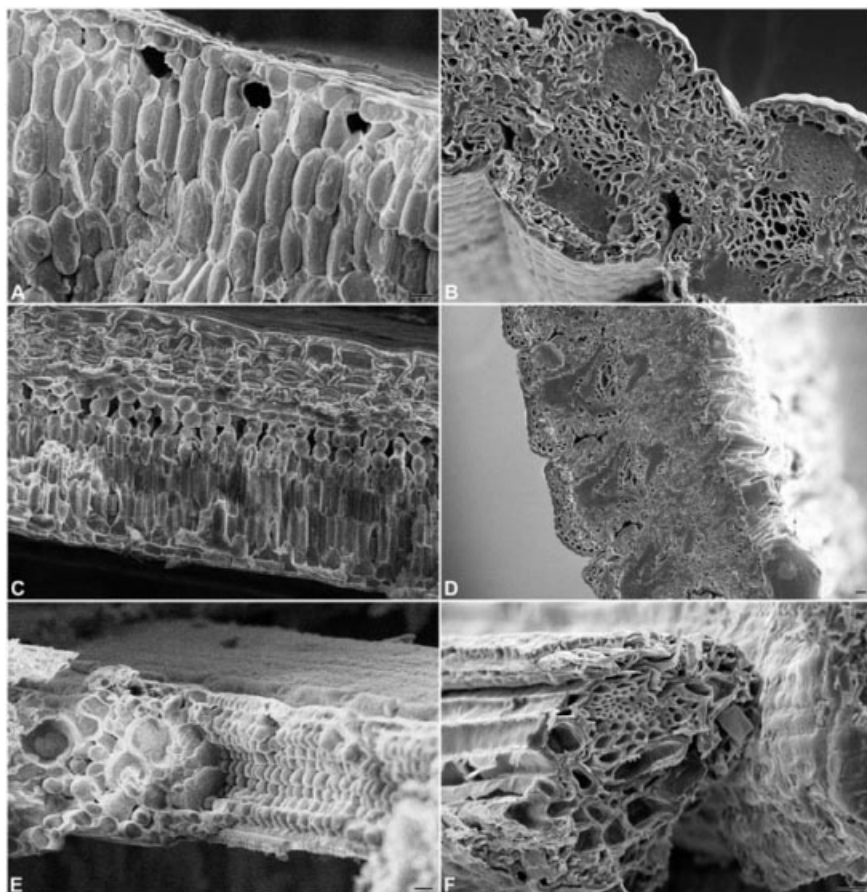


Fig. 3.7 Scanning electron microscopical images of hydrated (A,C,E) and dry ($\leq 5\%$ RWC [B,D,F]) leaves of the monocots *Xerophyta humilis* (A,B), *Xerophyta viscosa*, and *Eragrostis nindensis* (E,F). Scanning electron microscopy was performed using a Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo Transfer System. Leaves from hydrated and desiccated plants were frozen using liquid nitrogen and viewed directly or after freeze-fracturing. Scale bar for all images = 20 μm .

Thus, in those species using wall folding, there appears to be no uniformity among them in the manner in which reversible wall folding is achieved during drying. Indeed their overall wall composition is similar to other related DS species, but the resurrection species have utilized inherent wall characteristics, with only slight modifications during drying, to achieve stable and reversible conformational changes (Vicre et al. 1999, 2003, 2004a, 2004b, Moore et al. 2006). Comprehensive biochemical and immunocytological investigation of

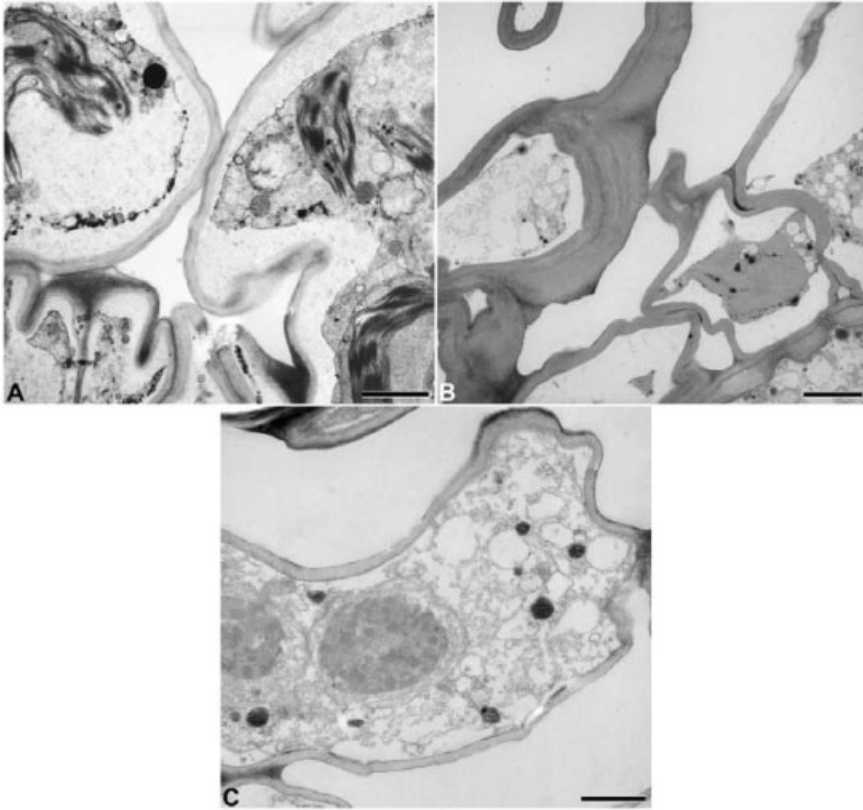


Fig. 3.8 Transmission electron micrographs of mesophyll leaves from dry leaves ($\leq 5\%$ RWC) of *C. wilmsii* (A), *E. nindensis* (B) and *S. stapfianus* (C) showing wall folding as a form of prevention of mechanical stress. Segments ($1\text{--}2\text{ mm}^2$) were excised from the mid-blade of dehydrated leaves and processed for TEM by the method of Sherwin and Farrant (1996). Microscopy was performed using a LEO 912 transmission electron microscope equipped with CCD camera. Scale bar for all images = $2\text{ }\mu\text{m}$.

leaf wall changes during drying and rehydration of *C. wilmsii* (Fig. 3.8A) has shown that the major difference between dry and hydrated walls lay only in the hemicellulose wall fractions (Vigre et al. 1999, 2004b). There was a reduction in glucose and an increase in galactose substitutions in the xyloglucans (XG) from dry walls compared with hydrated walls. We have proposed that cleavage, or partial cleavage of the long-chain XG units during drying into shorter, more flexible ones, allows for wall folding. SIMS revealed a marked increase in wall-associated Ca^{2+} but only at the final stages of drying. Because this ion plays an important role in cross-linking wall polymers, such as acid pectins, we propose that this serves to stabilize walls in the dry state and, more important, prevent mechanical stress of rehydration. *C. wilmsii* is a

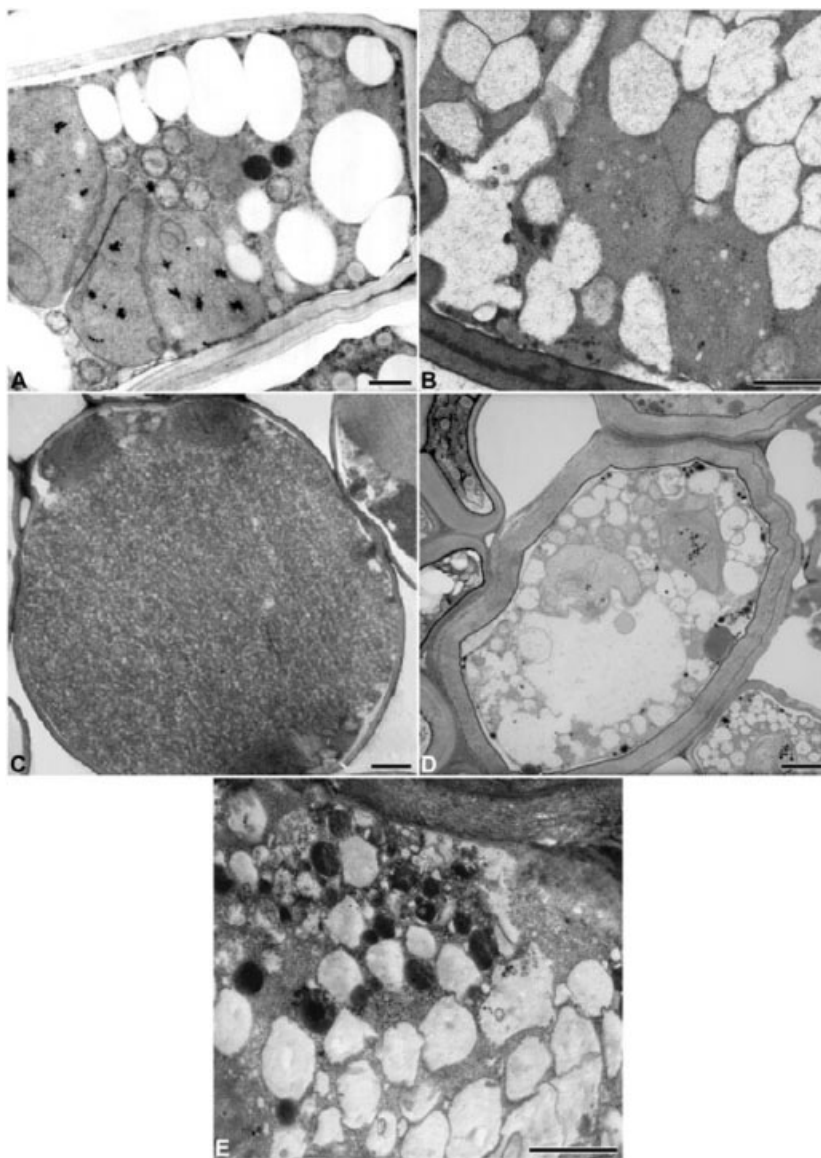


Fig. 3.9 Transmission electron micrographs of mesophyll leaves from dry leaves ($\leq 5\%$ RWC) of *X. viscosa* (A), *X. humilis* (B) *M. flabellifolius* (C) and bundle sheath cells of *E. nindensis* (D) and *S. stapfianus* (E) in which organelle packaging and vacuolation (with water replacement having occurred in vacuoles) serve as a means of prevention of mechanical stress. Segments (1–2 mm²) were excised from the mid-blade of dehydrated leaves and processed for TEM by the method of Sherwin and Farrant (1996). Microscopy was performed using a LEO 912 transmission electron microscope equipped with CCD camera. Scale bar for all images = 1 μ m.

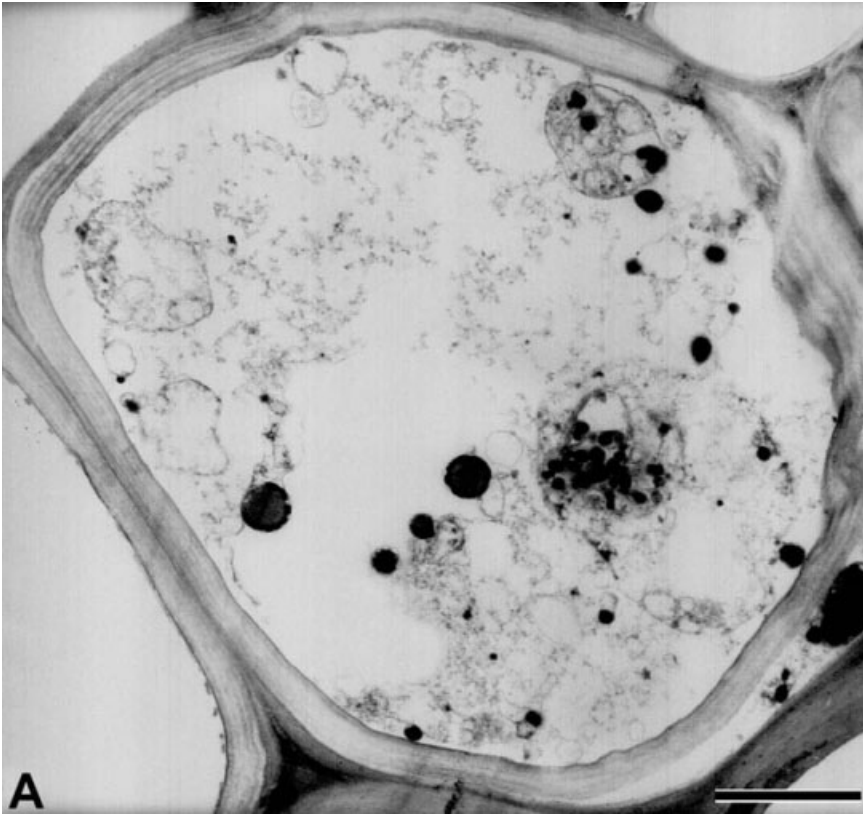


Fig. 3.10 Subcellular damage associated with desiccation in leaves of the DS species *E. curvula*. Note plasmalemma and tonoplast are disrupted and organelles are totally degraded. Scale bar = 2 μ m.

small plant, and rehydration is rapid and initially mainly apoplastic (Sherwin and Farrant 1996). If walls hydrate and unfold before cell volume is regained, plasmalemma tearing and further subcellular damage could occur (reviewed in Vire et al. 2003, 2004a). Jones and McQueen-Mason (2004) have shown an increase in abundance of an alpha-expansin transcript during drying and rehydration in leaves of *C. plantagineum* that correlated with changes in wall extensibility in that species. Expansins are proposed to be involved in wall loosening via disruption of noncovalent bonds between polysaccharides (McQueen-Mason and Crosgrave 1995), and this could be an additional or alternative mechanism whereby wall folding might be facilitated in the *Craterostigma* sp.

A similar biochemical, immunocytological study was conducted on leaf wall changes in *M. flabellifolius* (Moore et al. 2006). In this species, wall

folding occurs in the epidermis (around seemingly less flexible stomata and gland cells) and in the immediately adjacent mesophyll cells (Moore et al. 2007b) (Fig. 3.6D). The more centrally located mesophyll has less wall folding, and mechanical stabilization is almost entirely due to vacuole filling (Fig. 3.9C). In this species, there were no significant changes in wall components during drying, but walls contained an unusually high amount of arabinose, associated both with the pectins, presumably as arabinan polymers, and in arabinogalactin-rich wall proteins. Arabinose polymers are highly mobile and allow wall flexibility (Foster et al. 1996, Renard and Jarvis 1999) and have a high water-absorbing capacity (Goldberg et al. 1989, Belton 1997), which would be important for rehydration. We propose that arabinans are constitutively synthesized in leaf cell walls of *M. flabellifolius* and that their presence allows constant preparedness for dehydration–rehydration cycles in this species (Moore et al. 2006).

Wall folding occurs in mesophyll cells of the grasses *E. nindensis* and *S. stapfianus* (Fig. 3.8B and Fig. 3.8C, respectively), but the biochemical nature of wall changes has not been analyzed yet. In the bundle sheath cells of these species (Fig. 3.9D, E), as in mesophyll cells of the *Xerophyta* spp. (Fig. 3.9A, B) and *M. flabellifolius* (Fig. 3.9C), the large central vacuole present in hydrated tissues (not shown) is replaced by a number of smaller vacuoles, these serving to fill the cytoplasm, minimizing organelle compaction and membrane appression and preventing plasmalemma withdrawal. As is the situation for wall folding, while the general mechanism is common among species, the manner in which it is achieved varies among species and probably reflects modification of the metabolic characteristics already present in each species.

The content of these vacuoles has been analyzed biochemically for *E. nindensis* (Vander Willigen et al. 2004) and *M. flabellifolius* (Moore et al. 2005a, 2005b). Vacuoles were extracted and isolated from dry leaves of *E. nindensis* using nonaqueous extraction and density gradient protocols, and the vacuolar contents were analyzed biochemically. These were found to contain proline, sucrose, and protein in equal proportions (vander Willigen et al. 2004). A thorough biochemical analysis of *M. flabellifolius* (Moore et al. 2005a, 2005b, 2007b) revealed that the vacuoles (both hydrated and dry) contained the 3,4,5-tri-O-galloylquinic acid, and that this polyphenolic increased on drying to fill the vacuole (Fig. 3.9C) and stabilize the subcellular milieu against mechanical stress.

Because drying induces considerable changes in leaf subcellular structure due to these mechanisms of mechanical stabilisation, we asked whether this would have an impact on their leaf tensile properties. We used a portable tensiometer to compare the tensile properties of a range of resurrection plant species and found that while there were the standard differences among monocots and dicots as would be predicted from the literature (De Sousa et al. 1982,

Vincent 1982, O'Reagain 1993, Read and Sanson 2003, Balsamo et al. 2006), with the exception of the grasses, *E. nindensis* and *S. stapfianus*, there was nothing unusual in the tensile properties of the resurrection plant species, neither when wet nor dry (N. Algar, J. Farrant, R. A. Balsamo, unpublished data, Balsamo et al. 2006). Furthermore, their tensile properties were more closely related to DS relatives than to each other. This observation further supports the suggestion above that resurrection plants appear to utilize and adjust existing biochemical and metabolic makeup, rather than having evolved a common mechanism unique to DT species, to facilitate mechanical stabilization. Interestingly, though, when tensile properties of the resurrection grass *E. nindensis* were compared with three DS *Eragrostis* sp. with differing degrees of "drought tolerance" (Balsamo et al. 2006), we found that leaf tensile strength increased with degree of drought, but not desiccation, tolerance. Among the DS species, vascular bundle size and degree of lignification correlated with increased mechanical properties and extent of water loss tolerated before loss of viability. However, in the resurrection species *E. nindensis*, there was no change in tensile properties upon natural drying of the plants, despite the structural features of leaves of this species being similar to those of the significantly drought-tolerant *E. curvula* (Balsamo et al. 2005, 2006). The only remarkable difference among various *Eragrostis* sp. was the retention in air-dry ($\leq 0.1 \text{ g} \cdot \text{g}^{-1}$) *E. nindensis* of some water with apparently mobile properties as determined by proton NMR. We have proposed that this might explain the unusual tensile properties of the resurrection grasses (N. Algar, R.A. Balsamo, J. Farrant, unpublished data, Balsamo et al. 2006). It has traditionally been argued that at intracellular water concentrations $\leq 0.1 \text{ g} \cdot \text{g}^{-1}$ there is insufficient water to surround macromolecules and that whatever water is present would be tightly bound to macromolecular surfaces (Vertucci and Farrant 1995, Walters 1998, Hoekstra et al. 2001, Walters et al. 2002, 2005). However, by the argument outlined in the introduction to this chapter, it is highly likely that localized pockets of preferential hydration may occur in cells or even tissues. In this instance, we propose that this apparently mobile water in *E. nindensis* might be associated with the xylem tissue and may play a role in the prevention of embolisms and xylem cavitation.

Following on that subject, water transport through xylem imposes a physical constraint on DT in vascular plants (reviewed, e.g., by Sherwin et al. 1998, Proctor and Tuba 2002, Alpert 2005). Under most conditions and in all plants taller than about 3 m, water is believed to be pulled up through the xylem via cohesion forces due to transpirational loss at the leaf level. When the negative pressure in the xylem exceeds that which would serve to draw in an air bubble from a neighboring, air-filled conduit (as would occur under water deficit conditions), cavitation results and water flow is interrupted. Under conditions of severe water deficit, the water column continuity in xylem can be seriously compromised, and in air-dry plants, it is usually totally cavitared. Refilling of

empty xylem is thought to be feasible only in herbaceous species and small nonwoody shrubs. *M. flabellifolius* is the only resurrection plant with a woody stem, and the xylem is indeed severely cavitared during desiccation (Sherwin et al. 1998). Water columns are regenerated on rehydration, and this is believed to occur via a combined mechanism of root pressure (Schneider et al. 2000) and capillary action (Sherwin et al. 1998), the latter possibly being facilitated by an unusual lining of lipids on the inner surface of the xylem cell wall (Wagner et al. 2000, Schneider et al. 2003, Zimmerman et al. 2003), which appears to extend into the xylem parenchyma, acting possibly as a “wick” to facilitate rehydration from the xylem into the mesophyll (Moore et al. 2007b). Refilling may delay recovery from desiccation (Sherwin and Farrant 1996) and is likely to be impossible at heights greater than 3 m, resulting in a “desiccation pruning effect” in plants that have grown taller than this (Fig. 3.11).

3.1.3 Metabolic Stresses

As water is lost from the subcellular milieu, metabolism is increasingly perturbed, resulting in, *inter alia*, increasing free radical activity. Cellular contents become concentrated, increasing the chances of molecular interactions that can cause denaturation and membrane fusion, and ultimately, the lack of sufficient water to surround macromolecules will cause whole-scale subcellular denaturation. The ability to withstand such water loss requires adaptations that enable metabolic protection against these stresses.

3.1.3.1 Free Radical Stress (Reactive Oxygen Species). Free radicals are atoms or molecules with an unpaired electron, which is readily donated and thus highly reactive. Oxygen, albeit absolutely necessary for metabolism in all aerobic life forms, is a highly oxidizing molecule and readily forms other oxygen radicals such as singlet oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^{\bullet-}$), the hydroxyl radical ($\bullet\text{OH}$), and nitric oxide (NO^\bullet), collectively termed *reactive oxygen species* (ROS) (Halliwell and Gutteridge 1999). ROS are putated to cause damage to all macromolecules and subcellular components (reviewed in Hendry 1993, Pammenter and Berjak 1999, Mundree et al. 2002, Walters et al. 2002, Vicre et al. 2004, Berjak 2006, and elsewhere in this volume), and it is thus not surprising that ROS are frequently cited as being the most damaging consequence of desiccation stress (Hendry 1992, Smirnoff 1993, Kranner and Grill 1996, Kranner and Birtic 2005, Kranner et al. 2006). Because of their highly reactive nature, it is difficult to demonstrate ROS presence per se, and most often it is the accumulation of damage that is associated with ROS, together with the upregulation of antioxidants to quench their activity, that is given as proof of their villainous role in abiotic and biotic stresses. On the other hand, there is also convincing evidence for the role of ROS in intracellular signaling



Fig. 3.11 Desiccation pruning in *M. flabellifolius*. Desiccation causes xylem cavitation and refilling by root pressure and capillarity is effective only up to a height of 3m (Sherwin et al., 1998). Thus plants that are able to grow >3 m in the rainy season frequently cannot refill branch tips resulting in “desiccation pruning”. For color detail, please see color plate section.

(Finkel and Holbrook 2000, Apel and Hirt 2004, Bailly 2004, Laloi et al. 2004), and thus control of ROS for maintenance of subcellular homeostasis is also essential. While we have little information as yet on how these chemical species might play a role in signaling associated with DT, it is clear from our data reviewed later that angiosperm resurrection plants appear to go great lengths both to minimize metabolism that might result in excessive ROS formation and to quench their activity. It is also evident that the ability to maintain antioxidant potential during maintenance in the dry state is essential to recovery on rehydration (Farrant 2000, Illing et al. 2005, Kranner and Birtić 2005).

In all plants, ROS form as a natural consequence of metabolic processes involving electron transport, and thus mitochondria and chloroplasts are major sites of ROS production. Under hydrated conditions, their activity is neutralized and homeostatic control is realized by what has been referred to as the “classical” (Kranner and Birtić 2005) or “housekeeping” (Illing et al. 2005) antioxidants such as the water-soluble glutathione (γ -glutamyl-cysteinylglycine [GSH]) and ascorbic acid (Asc) (Noctor and Foyer 1998), the lipid-soluble tocopherols and beta-carotene (Munne-Bosch and Alegre 2002), and the enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP) and

other peroxidases, mono- and dehydroascorbate reductases, glutathione reductase (GR), and catalase (for an overview, see Elstner and Osswald [1994]). However, under severe water stress conditions, disruption of electron transport results in excess ROS production. While in seeds ROS accrue mainly from respiratory metabolism (Hendry 1993, Bailly 2004), in vegetative tissues the additional contribution from disruption of photosynthesis is critical. Excess excitation energy from chlorophyll rapidly results in formation of singlet oxygen, superoxide, and hydroxyl radicals (Halliwell 1987, Seel et al. 1992a, 1992b, Smirnov 1993). This excess ROS production is not adequately dealt with by DS plants, ultimately facilitating the loss of viability (reviewed in Hendry 1993, Smirnov 1993, Viret et al. 2003, 2004b, Bailly 2004).

Resurrection plants maintain respiration to low levels of RWC (Schwab et al. 1989, Hartung et al. 1998, Tuba et al. 1998, Farrant 2000, Vander Willigen et al. 2001, Mundree et al. 2002), giving a relatively large window of opportunity for unregulated ROS production. It is well documented that ROS activity can and does occur at low water contents, even at hydration levels I and II, in which tissues are considered to be in a glassy state (Farrant and Vertucci 1995, Walters et al. 2002, 2005), and we presume that antioxidant capacity, via both the ubiquitous “housekeeping” types and additional antioxidant processes (see later), are able to quench this associated ROS production in resurrection plants. Photosynthetically produced ROS, however, is minimized at high RWC (Tuba et al. 1998, Farrant 2000, Mundree et al. 2002, Farrant et al. 2003). In all the species we have examined, photosynthesis itself is switched off at water contents between 80% and 65%, depending on the species (Sherwin and Farrant 1998, Farrant 2000, vander Willigen et al. 2001, Mundree et al. 2002, Farrant et al. 2003, Illing et al. 2005) curtailing photosynthetically produced ROS in the first instance, which, together with upregulation of antioxidants (housekeeping and other), serves to minimize such ROS-associated damage. This downregulation of photosynthesis is achieved by two primary mechanisms: termed *poikilochlorophylly* and *homoiochlorophylly* (Gaff 1989, Smirnov 1993, Tuba et al. 1993, 1994, Sherwin and Farrant 1998, Farrant 2000).

Poikilochlorophyllous species, many of which are monocots and examples of which are the *Xerophyta* spp., *E. nindensis*, and *S. stapfianus* (Table 3.1; Fig. 3.5) break down chlorophyll and dismantle thylakoid membranes during dehydration (Sherwin and Farrant 1993, Tuba et al. 1993a, 1993b, Farrant 2000, Mundree and Farrant 2000). This strategy is highly effective in minimizing photosynthetically associated ROS production and has been proposed to be a major reason why poikilochlorophyllous species are able to remain viable in the dry state for far longer than homoiochlorophyllous ones (Tuba et al. 1998, J. Bajic and J.M. Farrant, unpublished data) (Table 3.1). The potential disadvantage of this strategy is the need to resynthesize the photosynthetic machinery de novo upon rehydration, thus retarding recovery rate. However, we have shown that in

X. humilis, RNA coding for the resynthesis of chlorophyll and reconstitution of thylakoids is transcribed during drying, is stably stored in the dry state, and is translated immediately on rehydration, even before reactivation of the nuclear genome (Dace et al. 1998, Collett et al. 2003).

Homoiochlorophyllous species, typically dicots, with examples of *Craterostigma* spp. and *M. flabellifolius* (Table 3.1; Fig. 3.1), retain most of their chlorophyll (the amount retained depends on the light levels under which the plants are dried) and thylakoid membranes in the dry state. They use various mechanisms to prevent the light–chlorophyll interactions that might cause ROS production during drying and rehydration (Sherwin and Farrant 1998, Farrant 2000, Farrant et al. 2003). This is achieved by leaf folding and shading of inner leaves (*Craterostigma* spp.) or adaxial surfaces (*M. flabellifolius*) from light (Fig. 3.1). In addition, there is accumulation of anthocyanin pigments (Table 3.2) in those surfaces that remain exposed to light in the dry state (Fig. 3.1); these supposedly act as “sunscreens,” reflecting back photosynthetically active light, masking chlorophyll and acting as antioxidants (Smirnoff 1993, Sherwin and Farrant 1998, Farrant 2000, Farrant et al. 2003, Moore et al. 2007a, 2007b). Homoiochlorophyllous species accumulate far more anthocyanins than do poikilochlorophyllous ones (Figs. 3.1 and 3.2; Table 3.2), affirming that these pigments may indeed play an important role in the prevention of ROS damage.

In addition to “avoiding” ROS production, resurrection plants, like DS types, also upregulate antioxidants to quench those ROS that are produced on drying. However, the difference between DT and DS species appears to be in (1) the ability to maintain oxidative potential of ubiquitous housekeeping antioxidants during dehydration and (2) the ability to produce, *de novo*, antioxidants that previously have been reported to occur only in seeds (Illing et al. 2005).

From the literature, there appears to be considerable variation between DT species with respect to the extent of upregulation of the various housekeeping antioxidants and the water contents during a dehydration–rehydration time course that the observed changes occur (reviewed, e.g., in Farrant 2000, Farrant et al. 2003, Illing et al. 2005). It is difficult to know whether this variation is real, because reporting in the literature is irregular. The conditions under which plants are dried vary; frequently, the water content to which the tissues are dried is not presented and/or the activity on rehydration is not recorded, or the manner of quantification differs. Furthermore, use of antioxidant concentrations alone has limitations, because they often show a Gaussian response to stress (Kranter et al. 2006), making interpretation of a single measurement ambiguous.

From the data on antioxidant enzyme activity collected in our laboratory where conditions were standardized and full dehydration–rehydration time courses were followed (Fig. 3.12), some of this variation appears real. All four antioxidant enzymes tested were active in hydrated tissues (i.e., they are truly

Table 3.2 Total phenolic content of leaves of resurrection plants and their antioxidant potential as determined by the FRAP¹ and DPPH² assays.

Resurrection Plant	Total Phenolics (mg GAE/g) DW	FRAP (mmol Fe ²⁺ /L)	% Inhibition of DPPH	Anthocyanin in Dry Leaf (μg.gdw ⁻¹)
<i>M. flabellifolius</i>	247.1 (15.9)	25.1 (0.8)	94.8 (0.4)	F 12 (0.9) D 32.2 (1.0)
<i>C. wilmsii</i>	47.9 (1.3)	11.5 (0.4)	47.7 (0.1)	F 11.9 (0.2) D 85.2 (2.9)
<i>C. plantagineum</i>	43.4 (5.1)	10.9 (0.4)	54.3 (1.3)	ND
<i>C. pumilum</i>	41.5 (2.3)	7.8 (0.2)	40.0 (1.4)	ND
<i>X. humilis</i>	38.9 (0.6)	7.7 (0)	31.7 (2.4)	F 13.0 (0.1) D 28.8 (1.1)
<i>X. viscosa</i>	39.6 (1.5)	8.0 (0.3)	36.1 (0.6)	F 12 (0.02) D 19 (0.14)
<i>X. schlechterii</i>	45.8 (5.1)	8.7 (0)	36.3 (1.5)	ND
<i>E. nindensis</i>	10.5 (1.1)	3.4 (0.1)	24.0 (2.6)	F 10 (0.1) D 99.2 (6.2)
Desiccation-Sensitive plants				
<i>E. curvula</i>	6.8 (1)	2.2 (0.2)	12.2 (1.0)	F 10 (0.2) D 12 (0.2)
<i>Aspalathus linearis</i> ³ (Rooibos tea)	255 (50)	19.5 (1.5)	ND	ND
<i>Cyclopia intermedia</i> ³ (Honeybush tea)	220 (18)	15.6 (2.2)	ND	ND

Extraction and quantification was as described in Farrant et al. (2007). Total soluble polyphenols were determined spectrophotometrically (Slinkard and Singleton, 1977) using gallic acid (GA) as a standard and the results expressed as mg GA equivalents per g dry weight (mg GAE/g DW). The free radical (electron) scavenging activities were evaluated by the DPPH² assay according to the method of Brand-Williams et al. (1995) and the FRAP assay by the method of Benzie and Strain (1996). Anthocyanins were extracted and quantified from dry leaves of plants using the method of Harborne (1998). F and D refer to levels in fully hydrated and dehydrated leaves respectively. Standard deviations are given in parentheses (n = 5).

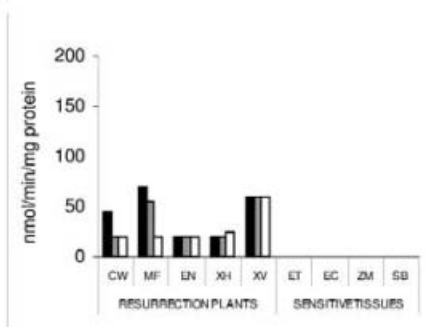
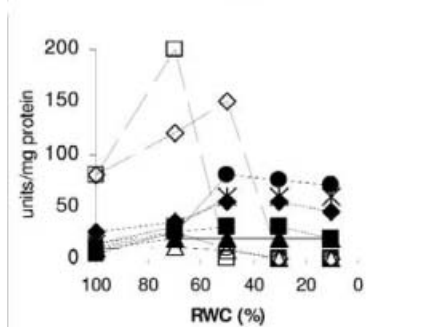
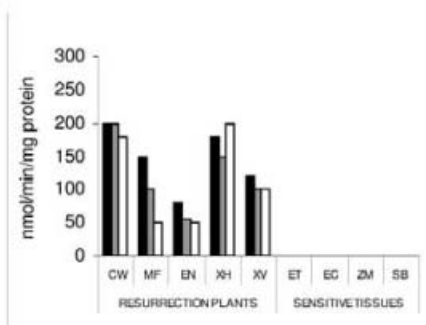
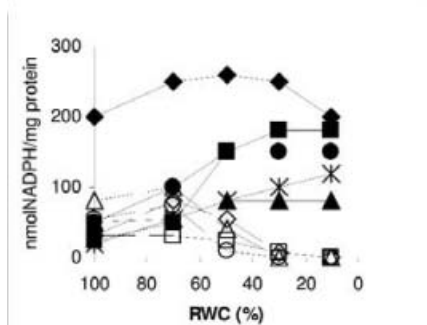
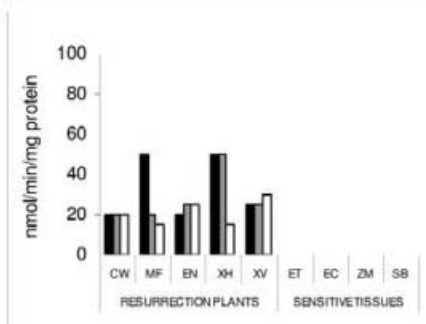
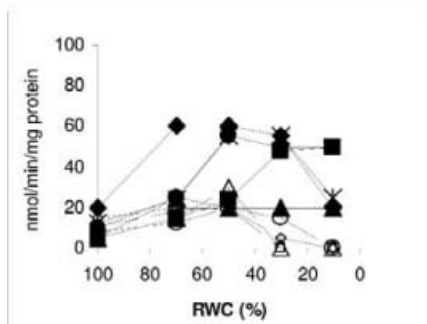
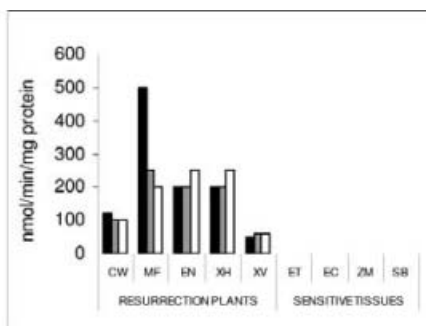
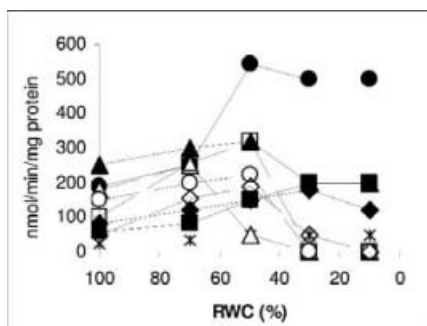
¹FRAP — ferric reducing/antioxidant power.

²DPPH 1.1 diphenyl-2-picrylhydrazyl.

³Taken from Somers (2002).

ND, not determined.

Fig. 3.12 (Right) Activities of the antioxidant enzymes ascorbate peroxidase (A,B), catalase (C,D), glutathione reductase (E,F) and superoxide dismutase (G,H) during dehydration (left panel, i.e. A,C,E,G) and rehydration (right panel, i.e. B,D,F,H). For the dehydration series the symbols are as follows: closed diamonds, *C. wilmsii*; closed circles, *M. flabellifolius*; closed squares, *X. humilis*; closed triangles, *Eragrostis nindensis*; stars, *X. viscosa*; open triangles, *E. teff*; open circles, *E. curvula*; open squares, *Zea mays*; open diamonds, *Sorghum bicolor*. For the rehydration series enzyme activities of dry (black bars), partially rehydrated (grey bars), and leaves that had recovered full turgor (white bars) are shown. None of the desiccation-sensitive species recovered enzyme activity upon rehydration when previously desiccated to 5% RWC. Antioxidant enzymes were extracted and quantified from leaf tissues at various stages of dehydration and rehydration using the protocols described in Farrant et al., 2004.



“housekeeping” antioxidants) in both DT and DS species, and all species were able to upregulate antioxidant enzymes on initial drying. But there were individual differences among species in the rate of activity of the various enzymes in hydrated tissues and in the degree of their upregulation in response to initial drying (Fig. 3.12, left). Importantly, however, only the resurrection plants were able to retain enzyme activity at lower water contents and through the rehydration to full turgor (Fig. 3.12, right). Presumably, the enzymes are protected from damage during desiccation in DT but not in DS plants (reviewed further later).

Kranner and Birtic (2005) and Kranner et al. (2006) have also postulated that maintenance of antioxidant potential, particularly of glutathione, is key to the survival of desiccation in a variety of DT systems. Those authors have demonstrated that the half-cell redox potential ($E_{\text{GSSG}/2\text{GSH}}$) can be used as a marker for plant stress and, more specifically, when $E_{\text{GSSG}/2\text{GSH}}$ exceeds -160 mV, stress becomes lethal and programmed cell death ensues. Interestingly, they have demonstrated that longevity of *M. flabellifolius* in the dry state was lost after 8 months when $E_{\text{GSSG}/2\text{GSH}}$ values became more positive than -160 mV (Kranner and Birtic 2005). These findings correlate well with our own longevity studies on *M. flabellifolius* (Farrant and Kruger 2001). Furthermore, we have shown loss of viability in dry stored *C. wilmsii* (3 months) and *X. humilis* (10 months, under the most adverse conditions) coincided with loss of activity of the antioxidant enzymes GR, catalase, and SOD, although $E_{\text{GSSG}/2\text{GSH}}$ did not become more positive than -160 mV (J. Bajic and J.M. Farrant, unpublished data). Because regeneration of GSH (and presumably other nonenzyme antioxidants such as ascorbate and tocopherol) is dependent on enzyme activity, survival of ROS activity ultimately must rest also on the ability to prevent enzyme inactivation during drying and early rehydration.

What is becoming increasingly apparent is that resurrection plants utilize additional antioxidants, such as 1- and 2-cys peroxiredoxins, glyoxalase I family proteins, zinc metallothioneine, and metallothioneine-like antioxidants (Blomstedt et al. 1998, Mowla et al. 2002, Collett et al. 2004, Illing et al. 2005), that have been reported to be important in DT of orthodox seeds but are never found to be upregulated in their (DS) vegetative tissues (Aarlen 1999, Stacey et al. 1999). Other unusual nonenzymic antioxidants proposed to facilitate protection against ROS are various polyphenols (Smirnoff 1993, Wang et al. 1996, Kahkonen et al. 1999). The resurrection plants we have studied have varying amounts of polyphenols; the potential antioxidant capacities are given in Table 3.2. In general, these are higher than those recorded for closely related DS species, and equivalent to our own calculation of antioxidant capacity of the commercial teas *Aspalathus linearis* (“rooibos”) and *Cyclopia intermedia* (“honeybush tea”) and the medicinal plant *Mellisa officianalis* (25.2 mmol Fe^{2+}/L) (Katalinic et al. 2005), which are valued for their antioxidant properties. Furthermore, leaves of *M. flabellifolius* contain a high

proportion (up to 50% of leaf dry weight) of 3,4,5-tri-O-galloylquinic acid, which we have demonstrated acts as a potent antioxidant in vitro (Moore et al. 2005a). Despite a predominant vacuole and cell wall location of this polyphenol, we suggest that these reservoirs act to absorb electrons from the cytoplasmically located housekeeping/ubiquitous antioxidants (i.e., redox-coupling), enabling them to retain their reducing potential in the face of severe water deficit. Indeed, there has been some research that indicates a potential link between the primary antioxidants in the Haliwell-Asada cycle and the vacuolar antioxidant plant polyphenols, even in DS plants (Takahana and Oniki 1997, Yamasaki and Grace 1998). The extreme quantities of such polyphenols in *M. flabellifolius* and other resurrection plants we have studied would greatly increase antioxidant potential in these plants compared to their DS relatives (Table 3.2).

As a final observation, it appears that the total antioxidant potential, if one combines the extent of upregulation of antioxidant enzymes (Fig. 3.12) and potential polyphenol antioxidant capacity and anthocyanin protection (Table 3.2), of the homoiochlorophyllous species (*M. flabellifolius* and the *Craterostigma* spp.), is greater than that of the poikilochlorophyllous types (*Xerophyta* spp. and *E. nindensis*). This supports the contention that homoiochlorophyllous resurrection plants might need better antioxidant protection against free radical activity than the poikilochlorophyllous ones, because the latter are able to better avoid ROS formation in the first place due to their dismantling of the photosynthetic apparatus (Tuba et al. 1998, Farrant 2000, Farrant et al. 2003).

3.1.3.2 Denaturation and Subcellular Perturbations. As water is progressively lost from the cytoplasm, it becomes viscous and molecular interactions occur that facilitate protein denaturation and membrane fusion. Clearly, these interactions, which start to occur at water concentrations of below 50% RWC or $0.3 \text{ g} \cdot \text{g}^{-1}$ (loss of type III and some of type II water), must be avoided in DT organisms (Vertucci and Farrant 1995, Walters 1998). Upon further water loss to 10% RWC, $\leq 0.1 \text{ g} \cdot \text{g}^{-1}$ (loss of type II and some type I water), the hydrophobic effect of water that is essential to the maintenance of macromolecular and membrane structure is lost and irreversible subcellular denaturation occurs. DT organisms clearly also survive loss of such structure-associated water. The theories on mechanisms whereby this is achieved come mainly from work done on model membrane systems and seeds and are largely thought to be due to the ability to substitute water with molecules that form hydrogen bonds that are able to stabilize macromolecular interactions in their native configuration (Crowe et al. 1986, 1987, 1998). In addition to this water replacement, further stabilization of the subcellular milieu is thought to be brought about by vitrification of the cytoplasm by the same candidates achieving macromolecular stabilization (Leopold 1986, Leopold et al. 1994,

Vertucci and Farrant 1995, Walters 1998, Hoekstra et al. 2001). The candidates for such replacement–stabilization reactions are given as (1) sugars, particularly sucrose together with oligosaccharides (reviewed, e.g., in Scott 2000, Illing et al. 2005, Berjak 2006); (2) proteins, particularly late embryogenesis abundant or LEA proteins (reviewed, e.g., in Illing et al. 2005, Mwtisha et al. 2006) and small heat shock proteins (Almogeura and Jordano 1992, Wehmeyer et al. 1996, Mtwisha et al. 2006); and (3) various other compatible solutes, including amino acids such as proline (e.g., Tymms and Gaff 1978, Gaff and McGregor 1979) and amphiphiles (Golovina et al. 1998, Golovina and Hoekstra 2000, Hoekstra et al. 2002). While glass formation has been reliably demonstrated to exist in dry systems and is quite widely accepted as a plausible mechanism for subcellular stabilization *in the dry state* (Walters 1998, Buitink et al. 2002, Walters et al. 2005), the theory of water replacement is becoming a widely debated subject. Such mechanistic details are beyond the scope of this chapter and they are excellently covered elsewhere in this volume (see, e.g., Chapter 6). What is relevant here is to review the extent to which these candidates play a role in DT of angiosperm resurrection plants, and this is reviewed later.

3.1.3.2.1 Sugars. Sucrose is apparently universally accumulated in leaves and roots of all angiosperm resurrection plants examined to date (Bianchi et al. 1991, Ghasempour et al. 1998, Norwood et al. 2000, 2003, Bartels and Salamini 2001, Whittaker et al. 2001, 2004, Illing et al. 2005, Peters et al. 2007) (Fig. 3.13). What is equally characteristic is that sucrose accumulation occurs relatively late in the dehydration time course, initiated usually below leaf RWC of 60%, but in some, such as *X. humilis*, the majority of accumulation occurs at 20% RWC or less (Fig. 3.13). This accumulation is generally after photosynthesis has been switched off (Mundree et al. 2002, Illing et al. 2005), and thus the source of carbon for sucrose accumulation has been debated. In *C. plantagineum*, it has been shown that octulose and stachyose decline in leaves and roots, respectively, as sucrose accumulates, and it has been postulated that those oligosaccharides are converted into sucrose during drying (Norwood et al. 2000, 2003). Sucrose is also universally accumulated in orthodox seeds (Amuti and Pollard 1977, Koster and Leopold 1988, Vertucci and Farrant 1995, Pammenter and Berjak 1999, Berjak 2006), and this evidence, correlative as it might be, provides rather convincing evidence that it is this sugar that plays an important role in DT in plants in general. Our data on localization of sucrose show that it is mainly cytoplasmic, predominantly in mesophyll and cortical parenchyma of leaf and root tissues, respectively (Fig. 3.14). It is a minor constituent of vacuoles in those species in which water replacement in vacuoles occurs during drying (vander Willigen et al. 2004). We propose that this ubiquitous presence of sucrose plays an

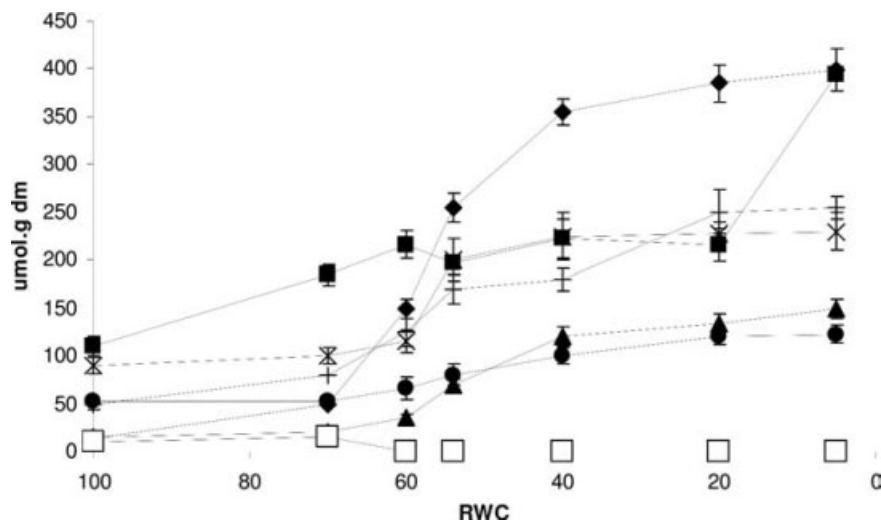


Fig. 3.13 Changes in leaf sucrose content during drying of resurrection plants *C. wilmsii* (closed circles), *M. flabellifolius* (closed squares), *X. humilis* (closed triangles), *X. viscosa* (stars), *Eragrostis nindensis* (open circles); *S. stapfianus* (crosses), and the DS species *E. curvula* (open squares). Sucrose was extracted from leaves and quantified as previously reported in Illing et al., 2005.

important role in glass formation and stabilization of the subcellular milieu during maintenance in the dry state.

As is the situation in seeds, oligosaccharides do accumulate during drying in resurrection plants, but the nature and extent of oligosaccharide accumulation vary and in no instance is the accumulation during drying greater than that of sucrose (Table 3.3). Interestingly, trehalose, the sugar that is proposed to be an exceptional membrane stabiliser (Kaushik and Bhat 2003) and as a water replacement molecule in animal systems (Crowe et al. 1986, 1987, 1998), accumulates to any great extent only in *M. flabellifolius*, and the extent of accumulation in this species is insufficient to serve either proposed function. It is widely held in the seed literature that the raffinose series oligosaccharides (RFOs), particularly raffinose and stachyose, may play an important role in stabilization of the subcellular milieu by either water replacement or vitrification (for reviews, see, e.g., Buitink et al. 2002, Kermode and Finch-Savage 2002), and it is indeed these two sugars that are most commonly accumulated in the resurrection plants we have examined to date (Table 3.3). However, the variability in amounts accumulated (or not) is such that we consider that oligosaccharides and various compatible solutes may serve interchangeably in affording protection and that the particular metabolite accumulated is species specific and reflects the predominant metabolism associated with the hydrated condition. The protection functions they could serve are (1) facilitation of glass formation, as well as probably preventing sucrose

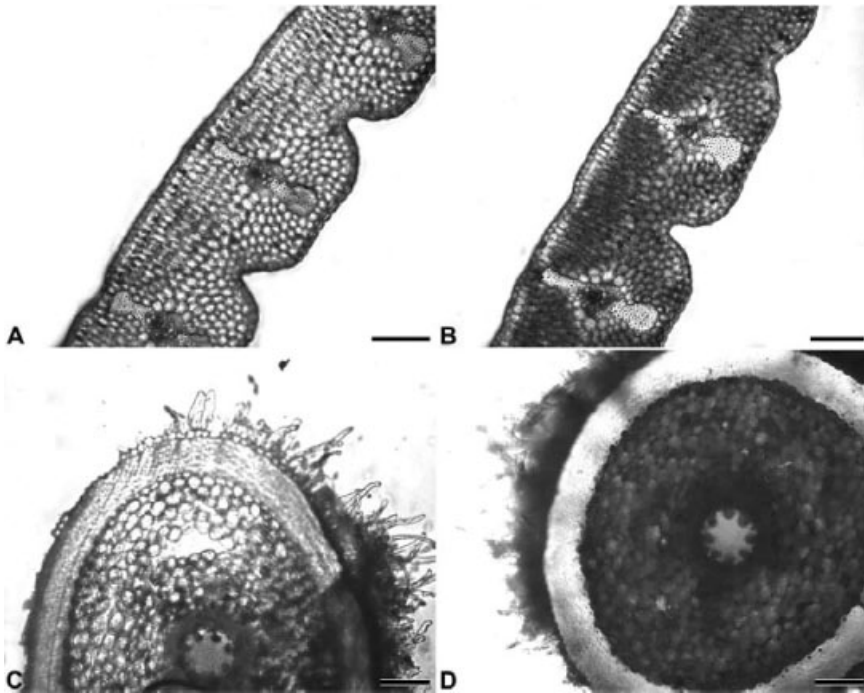


Fig. 3.14 Sucrose localization (B,D) in hand cut, unfixed, cross sections of dehydrating leaves (A,B) and roots (C,D) of *X. humilis*. Leaf RWC is 20% RWC (0.25 g.g^{-1}) and root RWC is 10% (0.12 g.g^{-1}). Sucrose visualization was using the colorimetric method of Martinelli (2007) in which the presence of sucrose (panels B,D) is identified by the red formazan precipitation from the reduction of tetrazolium (iodonitrotetrazolium chloride) upon coupling of a reaction in which sucrose is the substrate. Control sections (A,C) were not exposed to INT. Sucrose is mainly cytoplasmic. Scale bar for all images = 200 μm . For color detail, please see color plate section.

crystallization in the glasses; (2) filling of vacuoles in those species that use this means of mechanical stabilization; (3) removal of monosaccharides in the process of their formation; and (4) an additional carbon source for recovery during rehydration. In many species, however, the oligosaccharide content declines during drying as is almost universally the case for the monosaccharides (Vertucci and Farrant 1995, Walters et al. 2002) (Table 3.3). The loss of oligosaccharides can be due to the use of their C skeletons for the formation of sucrose. The reduction in monosaccharides during drying is thought to limit respiration and the associated ROS production and to induce metabolic quiescence required in the desiccated state (Vertucci and Farrant 1995, Farrant et al. 1997). Furthermore, because monosaccharides participate in Maillard-type reactions and because binding to proteins can cause their glycation, their

Table 3.3 Contents of various saccharides in hydrated and dry leaves of various resurrection plants.

Species	Tre	Oct	Raf	Stach	Suc	Fru	Glu
<i>C. wilmsii</i>	F	ND	0.5 (0.01)	5.6 (0.5)	13 (0.3)	92 (5)	112 (2)
	D	ND	2.5 (0.02)	16.6 (0.8)	400 (13)	4 (0.1)	2.2 (0.2)
<i>C. plantagineum</i> LEAVES	F	ND	Not reported	Not reported	2000	104.2	105
<i>C. plantagineum</i> ROOTS	D	51		reported	73	8	135
	F	61.9 ± 10	82.5 ± 2.9	614 ± 20	36.9 ± 7.7	0 ± 0	4.2 ±
<i>M. flabellifolius</i>	D	4.9 ± 0.7	36.9 ± 0.5	259 ± 16	111 ± 8	12.2 ± 0.6	10.6
	F	ND	0.4 ± 0.2	7.4 ± 2.7	52 ± 1	113 ± 5	73 ±
<i>E. nindensis</i>	D	45.8 ± 2	4.8 ± 1.6	2.7 ± 1.5	123 ± 10	39 ± 4	67 ±
	F	70 ± 5	0.0 ± 0	0 ± 0	15 ± 0.1	1.6 ± 0.1	4.6 ±
<i>X. viscosa</i>	D	1.0 ± 0.14	3.0 ± 0.04	1.63 ± 0.09	150 ± 12	9.4 ± 0.1	6.8 ±
	F	1.2 ± 0.16					
<i>X. villosa</i>	D	ND	9.9 ± 0.2	3.6 ± 0.2	90 ± 8	10 ± 0.2	18 ±
	F	ND	39.4 ± 2	26.5 ± 0.5	230 ± 11	4 ± 0.02	5 ±
<i>B. constricta</i>	D	ND	11.2 ± 0.5	12.5 ± 0.1	43 ± 0.7	5.2 ± 0.1	3.1 ±
	F	ND	7.16 ± 0.2	4 ± 0.3	82 ± 0.9	0.6 ± 0.02	1.2 ±
	D	0.4 ± 0.03	0.73 ± 0.05	0.66 ± 0.01	33 ± 0.3	40 ± 1	23 ±
	F	0.2 ± 0.02	5.1 ± 0.06	4.43 ± 0.05	44 ± 0.3	11 ± 0.1	6.4 ±

F = fully hydrated leaves; D = air dry leaves. Sugar contents expressed as $\mu\text{mol.g.dwt}^{-1}$. Mechanisms of extraction and quantif given. ND not detected.

removal during drying can limit these damaging reactions (Vertucci and Farrant 1995, Mtwisha et al. 2006).

3.1.3.2.2 LEAs. As the name suggests, late embryogenesis abundant proteins were first identified due to their abundant (4% of total cellular protein, Roberts et al. 1993) accumulation during the late stages of seed development coincident with the onset of DT (Galau et al. 1986, Blackman et al. 1992, 1995, Baker et al. 1995, Russouw et al. 1995, Manfre et al. 2006). They have subsequently been found to be widespread among prokaryotes (Garay-Arroyo et al. 2000) and eukaryotes, where they are apparently less prevalent in the animal kingdom (but found, e.g., in nematodes and tardigrades [Goyal et al. 2005]), but they are almost ubiquitously present in the plant kingdom (e.g., Stacy et al. 1995, Close 1996, 1997, Bray 1997). In plants, they appear to be expressed predominantly in response to various abiotic stresses (desiccation, cold, drought, salt and osmotic), but at least four of the *Arabidopsis* LEAs (At1g54410/LEA2, At5g53820/LEA3, At4g02380/LEA7, and At5g18979/LEA10) have been identified as being constitutively present “housekeeping” proteins (Illing et al. 2005).

Their nomenclature, at present, is confusing. This is largely because since their initial discovery in the 1980s, a great number have been independently reported by different authors, many ascribing their own nomenclature or label. The situation is further complicated by the fact that they are low-complexity proteins that are largely unfolded in the hydrated state, making it experimentally difficult to assign structure and determine potential function, for which they can be classified and named. However, Bray (1993, 1997) has attempted to classify them into at least five groups dependent on their amino acid sequences, and more recently, Wise (2003) and Wise and Tunnacliffe (2004) proposed up to nine groups (superfamilies) based on Protein or Oligonucleotide Probability Profiles (POPP)—essentially from an analysis of peptide composition of the proteins rather than sequence similarities. Berjak et al. (see Chapter 6) attempt to make sense of the nomenclature, but for the purposes of this chapter, I will use the Wise (2003) nomenclature LEA superfamilies.

As already mentioned, there is as yet little understanding of the exact role of LEAs—largely because few people work on the actual proteins themselves (some exceptions being Russouw et al. 1995, 1997, Mtwisha et al. 1998, Wolkers et al. 2001, Goyal et al. 2005), most of the work being based on RNA sequence characterization and predictive protein functions. These predicted functions, based on their rich hydrophilic amino acid content and their thermostability, are (1) as water replacement molecules or hydration buffers, (2) ion sequesters, (3) chaperonins, (4) prevention of protein and membrane aggregation, and (5) together with sugars, facilitating glass formation (Bray 1997, Vicre et al. 2003, 2004a, Berjak 2006, Mtwisha et al. 2006). While I

have no definitive proof at yet, I believe that there will be subsets of LEAs within the superfamilies (particularly LEA-1 but also LEA-4, LEA-6, and LEA-9, that are strongly seed specific in *Arabidopsis* [Illing et al. 2005]) that are specifically involved in desiccation and minimally (if at all) in other abiotic stresses, including drought. Thus, we are interested in whether the LEAs in resurrection plants are specifically induced only upon desiccation stress and whether these are similar among resurrection plant species and common to those induced during DT in seeds.

Reports on LEAs in resurrection plants, ours included, to date are based almost entirely on transcriptome studies and are summarized in Table 3.4. Although the table reflects only a small number of reported LEAs, this is probably because of the relatively small scale of the experiments done, as well as the lack of ability to correctly annotate some “unknown” transcripts as LEAs. We have reported 16 LEA transcripts to be upregulated during drying in leaves of *X. humilis*. This was from a minimicroarray experiment in which only 424 cDNAs from an 11k normalized (6% redundancy) root and leaf cDNA library were arrayed, sequenced, and annotated, and in which only 55 were significantly desiccation upregulated (Collett et al. 2004). While we cannot as yet tell if these LEAs are common to those reported in other resurrection plants, there was at least one LEA-6 and a LEA-4 that are specific to seed DT and maturation, respectively (Table 3.4). Our recent unpublished work has identified three additional candidates of the LEA-1 superfamily in seeds and desiccated leaves and roots of *X. humilis* that are seed specific in *Arabidopsis*, barley, and wheat (Stacy et al. 1995, Vicient et al. 2000, Manfre et al. 2006). Importantly, the transcripts for these proteins are evident only once the plant RWC drops below 50% and disappear during early rehydration ($\text{RWC} \leq 40\%$) (Illing et al. 2005 and unpublished observations) (Table 3.4). Clearly, these proteins play an important role in DT. In other studies on resurrection plants, two (from a differential screen of only 30 cDNAs) were reported to be desiccation induced in *X. viscosa* (Mundree and Farrant 2000, Ndimba et al. 2001), two from *S. stapfianus* (Blomstedt et al. 1998) and four were upregulated in leaves of *C. plantagineum* (Piatkowski et al. 1990). Further characterization of these LEAs at the protein level is required, and function ascribed, before we can know if there are commonalities between resurrection plants and indeed among resurrection plants and seeds (or other DT systems). I started this section saying that resurrection plants must survive the stresses associated with loss of type III and II water and that sugars, LEAs, and various compatible solutes may play a role in preventing them. How this is achieved is not yet clear, and the traditional theories might well prove correct. However, I venture here to make some observations from our work reported above that might negate some predictions and reinforce others. The massive accumulation of sucrose (Fig. 3.13), oligosaccharides (Table 3.3), and LEAs (Table 3.4) in leaves of resurrection plants occurs at RWC between 50% and 10% when the rate of

Table 3.4 Summary of LEA's reported to be desiccation-upregulated in leaves of resurrection plants.

Species	LEA Superfamily (Wise, 2003)	Genbank Accession #	Description	Log ₂ FH/D Ratios and RWC at Significantly Upregulated
<i>C. plantagineum</i>	LEA-3	P23283	Desiccation-related protein [<i>C. plantagineum</i>]	NA
	LEA-8	P22241	Desiccation-related protein [<i>C. plantagineum</i>]	NA
	LEA-2	P22238	Desiccation related protein [<i>C. plantagineum</i>]	NA
	LEA-2	S43775	Desiccation related protein [<i>C. plantagineum</i>]	NA
	LEA-2	CK906385	none	3.17 27%
<i>X. humilis</i>	LEA-2	CK988413	44 kDa dehydrin-like protein [<i>C. sericea</i>]	4.55 48%
	LEA-2	CK906432	Embryonic abundant protein—radish	2.49 48%
	LEA-2	CK906386	Dehydrin-like protein [<i>M. sativa</i>]	1.72 60%
	LEA-3	CK906406	LEA-like protein [<i>L. longiflorum</i>]	4.12 48%
	LEA-3	CK906427	LEA-like [<i>A. thaliana</i>]	1.96 48%
	LEA-3	CK906404	LEA protein 76—rape	2.18 48%
	LEA-3	CK906402	LEA1 protein [<i>T. aestivum</i>]	4.51 48%
	LEA-3	CK906398	LEA protein [<i>B. inermis</i>]	2.48 48%
	LEA-4	CK906399	Putative seed maturation protein [<i>O. sativa</i>]	1.58 48%
	LEA-6	CK906408	Seed maturation protein PM26 [<i>G. max</i>]	1.8 48%
	LEA-7	CK906401	LEA homolog—tomato	1.4 27%
	LEA-8	CK906400	LEA protein Lea 14-A upland cotton	4.78 48%
	LEA-10	CK906403	LEA protein with hydrophobic domain [<i>G. max</i>]	2.17 48%
	LEA-10	CK906405	Hydrophobic LEA-like protein [<i>O. sativa</i>]	2.7 60%
	LEA-10	CK906407	Putative plasmamembrane associated protein [<i>O. sativa</i>]	2.83 60%
<i>X. viscosa</i>	LEA-2	AAP22171	protein [<i>O. sativa</i>]	42%
	LEA-2	NA	<i>Xerophyta viscosa</i>	
<i>S. stapfianus</i>	LEA-2	EMBL:Y10778	Dehydrin	NA 23–27%
	LEA-3		LEA-like protein—wheat	NA 57%
		Y10779		

* Average log₂ expression ratios, fully hydrated (FH) vs dehydrated (D) leaf tissue from microarray experiment described in Illing et al., parentheses = standard deviation

** RWC at which mRNA significantly upregulated from reverse Northern blot experiments described in Collett et al., 2004.

dehydration is at its greatest (Fig. 3.3) and stomata open to apparently actively release of water from leaf tissue (Vicre et al. 2003, 2004a, Moore et al. 2007a, 2007b) (Fig. 3.4). In *X. humilis*, for example, there is a 200-fold increase in sucrose and between 6- and 14-fold increase in various stress-associated transcripts, including LEAs (Collett et al. 2004, Illing et al. 2004), as 60% of the RWC is lost in a period of 12 hours. It is possible that the rapidity with which this happens facilitates the trapping of water shells around macromolecules, maintaining them in native configuration, as has been previously proposed to occur at intermediate water contents of $0.3 \text{ g} \cdot \text{g}^{-1}$ (Hoekstra et al. 2001). But, I propose that such water shells (a few layers at most) may be retained by a coating of outer proteins and sugars, rather than the sugars and/or proteins themselves ultimately taking over the role of water replacement.

Such water would have different characteristics from bulk water (or not, as we have reported in *E. nindensis* [Balsamo et al. 2005]), and is possibly not recorded as “water” when using the traditional method of oven-drying at 70°C for 48 hours. Such a mechanism would prevent macromolecular and membrane fusion and prevent enzyme denaturation (as we show for the antioxidant enzymes in Fig. 3.12) but would not prevent the ultimate vitrification of the cytoplasm, which would happen as sugars, LEAs, and compatible solutes further increase and bulk water is lost. Ultimately, the dry state is likely to be stabilized by the formation of high-viscosity, high-stability glasses composed of sucrose, RFOs, and proteins, as has been variously proposed and demonstrated (Leopold et al. 1994, Walters 1998, Wolkers et al. 1998, 2001, Hoekstra et al. 2001, Buitink et al. 2002).

3.2 Concluding Statements

The work outlined here indicates that there are some key differences among resurrection plants in their responses to desiccation but also some unequivocal similarities, particularly at the biochemical level. It is my belief that with the advent of more transcriptome, proteome, and metabolome studies, these similarities will become increasingly apparent. For example, we are interested in the evolution of DT in vegetative tissues and have proposed that it is derived from an appropriation of the program of gene expression in orthodox seeds into the vegetative tissues (Illing et al. 2005). To further elucidate this proposition, we have conducted microarray studies to characterize differential gene expression patterns in hydrated and dry tissues of roots and leaves and to compare these with gene expression in seeds of *X. humilis* (Illing et al. 2006, S. Walford, J.M. Farrant, N. Illing, unpublished data). We have identified a cohort of genes that are commonly expressed only in the dry state of all three tissue types. A further cohort are expressed in seeds, dry leaves, and dry roots

but are also constitutively expressed in hydrated roots (probably because this is where water deficit is first sensed in plants).

These data support our contention that DT in vegetative tissues is possibly an activation of the seed desiccation program, but to environmental rather than developmental cues. It also underscores the probable commonality of many of the mechanisms of DT, because the mechanisms in orthodox seeds are presumed to be universal (Bewley and Black 1994, Vertucci and Farrant 1995, Pammenter and Berjak 1999, Berjak 2006, Mtwisha et al. 2006).

DT is a complex phenomenon and involves a great deal more than what is outlined here. We know little about the control mechanisms involved, from the environmental sensing of water deficit to the pre- and post-transcriptional and -translational control. We need a greater understanding of the full spectrum of protectant metabolites involved and of the role of repair mechanisms, during both drying and rehydration. To date, more focus has been placed on mechanisms of DT in leaves than in roots, and we need to start gaining an understanding of the whole plant integrative responses to desiccation. While transcriptome studies have gone some way in advancing our understanding of DT, these, together with proteomic and metabolomic studies, will enable us to eventually glean a clearer picture of the phenomenon as it pertains to the vegetative tissues of angiosperm resurrection plants.

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4 Desiccation Tolerance in Lichens

Richard P. Beckett and Farida V. Minibayeva

4.1 Introduction

Lichens are symbiotic organisms, comprising a fungus (the mycobiont) and either an alga or a cyanobacterium (the photobiont). The dual nature of the lichen symbiosis was first discovered by Schwendener in 1867. They range in size from tiny crusts less than 1 mm² to pendulous forms that hang more than 2 m from tree branches. Lichenization is one of the most successful ways that fungi use to fulfill their need for carbohydrates, and about 20% of all fungi are lichenized (Hawksworth 1988). Hawksworth and Hill (1984) estimate that there are 13,500 species of lichens in the world, corresponding to 15% of all fungi. However, these obligatory symbiotic species are not randomly distributed within the fungal kingdom. Lichenization is almost restricted to the Ascomycota (ascolichens), which claims more than 98% of all lichen-forming species (Hawksworth 1988); 42% of the Ascomycota are lichenized. Interestingly, recent phylogenetic analyses based on DNA sequences suggest that major lineages within the Ascomycota are derived from lichen symbiotic ancestors (Lutzoni et al. 2001). The remaining lichen-forming fungi are mostly members of the Basidiomycota (basidiolichens), representing less than 2% of the lichen diversity.

Although lichens represent a minor component of most terrestrial ecosystems, they form the dominant plant life on 8% of the world's land surface, mainly in arctic and antarctic regions (Ahmadjian 1995). These habitats are characterized by severe abiotic stresses such as desiccation, temperature extremes, and high light intensities. For this reason, lichens have been called "extremophiles," organisms that can thrive in conditions that would kill other, less-specialized organisms. Scientists have found that lichens can survive a trip into space—during a recent experiment by the European Space Agency, lichens were placed onboard a rocket and launched into space, where they were exposed to vacuum, extreme temperatures, and ultraviolet radiation for 2 weeks. Upon analysis, it appeared that the lichens survived with minimal damage (Young 2005).

The aim of this review is to present a general overview of our current knowledge about desiccation tolerance in lichens. For other recent reviews on desiccation tolerance in lichens, the reader is referred to Kranner and Lutzoni (1999) and Nash et al. (2006). It is important not to forget that desiccation is

often accompanied by other stress factors such as high temperature and high light intensity. Furthermore, at the cellular level, many stresses may have the same effects, such as the production of reactive oxygen species (ROS) and damage to the cytoskeleton. In lichens, the underlying mechanisms of tolerance to various stresses probably share many common features with each other, and with those in other organisms.

4.2 Limits to Desiccation Tolerance

Because lichens grow so slowly, it is difficult to use growth to assess the effects of desiccation. Lichenologists tend to measure parameters such as net photosynthesis or chlorophyll fluorescence to determine stress effects in the photobiont, while if they are more interested in the mycobiont they may study respiration or leakage of intracellular soluble potassium through membranes. The life of most lichens is characterized by rapidly changing water contents and correspondingly rapidly changing physiological activity such as respiration and photosynthesis (Kappen 1988). Variations in precipitation (rain, snow, or fog) and dewfall largely account for the varying hydric conditions of the lichens and their corresponding CO₂ exchange activity. For example, lichen mats (≈ 10 to 15 cm deep) occupy vast areas of Subarctic and Arctic regions in the Northern Hemisphere. Only the upper few centimeters of these mats are photosynthetically active (Nash et al. 1980), and these tips exhibit rapid drying and wetting cycles with changing microclimatic conditions (Moser et al. 1983, Lange et al. 1998). During clear periods, the physiologically inactive bases of the mats may remain moist, but the tops are desiccated. Similar patterns are documented for Antarctic lichens (Kappen 1988) and many temperate-zone lichens, such as *Lecanora muralis* on a rock wall in Würzburg, Germany, the subject of Lange's (2002, 2003) year-long investigation. To survive, it is clear that most lichens must be desiccation tolerant.

Early workers determined the limits of desiccation tolerance in lichens, and the conclusion of Kappen's (1974) excellent review of this work is that most lichens are highly desiccation tolerant. Providing that desiccation occurs reasonably slowly (over hours rather than minutes), most lichens can withstand drying to water contents of 5% or less, and most can remain viable for months, providing they are stored at low relative humidities. Even aquatic species such as *Dermatocarpon fluviale* can survive desiccated for 4 weeks. Conversely, most lichens are highly intolerant of submergence, or in many cases even moist storage, for more than a few days. Such lichens appear to become overrun with pathogenic fungi or dissociate into separate symbionts. While many lichenologists have tended to carry out their experiments on desiccation stress at room temperature, recent work on seeds suggests that a strong interaction

exists between temperature and storage time (I. Kranner, unpublished data). In other words, the ability to survive desiccation greatly increases as temperature decreases. We may have to revisit some of the earlier work on lichens—we might be surprised how tough lichens really are!

4.3 Harmful Effects of Desiccation Stress

Black and Prichard (2002) rightly pointed out that most studies on desiccation in plants tend to focus on how organisms tolerate stress, rather than trying to quantify the effects of stress. Actual studies documenting precisely how stress damages lichens are rare. However, based on studies with other organisms, it seems likely that desiccation will damage the cytoskeleton, make membranes leaky, and change the structure of proteins so that, for example, the activity of enzymes is reduced. One feature shared by almost all stresses is that they cause the formation of reactive oxygen species (ROS). Many ROS are free radicals, atoms, or molecules with an unpaired electron. This unpaired electron is readily donated, and as a result, most free radicals are highly reactive. Oxygen radicals include superoxide ($\text{O}_2^{\bullet-}$), the hydroxyl radical (OH^\bullet), and the nitric oxide radical (NO^\bullet). Hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$) are technically not free radicals but are nevertheless highly reactive, and together with the above-mentioned radicals are usually classified as “ROS.” It is important to realize that while small quantities of ROS are produced as byproducts of oxygen-dependent reactions during normal metabolism, almost all stresses enhance their production. Intracellularly produced ROS can cause considerable damage to cells by attacking nucleic acids, lipids, and proteins. To survive desiccation, lichens must be able to either reduce the formation of ROS, or detoxify them once formed. Furthermore, the reactions resulting from the detoxification of ROS can considerably change the cellular redox potential of lichens (Kranner 2002). ROS and redox potentials are now believed to play important roles in cell signaling (Hancock 2005), and it seems inevitable that the signaling systems of lichens will undergo regular disruption every time that a thallus becomes desiccated. Given the importance of ROS in desiccation damage and tolerance, the first part of this review focuses on the ways in which lichens control the concentrations of ROS in their tissues, while the second part focuses on other mechanisms of desiccation tolerance.

4.4 Controlling Reactive Oxygen Species Metabolism Is Important in Desiccation Tolerance

The effects of ROS are not all negative; for example, as discussed, they also play important roles in signaling processes. They may also be required for

processes such as melanization, delignification, and pathogen defense (Laufer et al. 2006). However, stress will arise if pro-oxidative processes prevail. Therefore, desiccation tolerance requires mechanisms that keep ROS under control.

4.4.1 *Measuring Reactive Oxygen Species Formation*

Measuring the production of ROS by lichen tissues is difficult because of their short half-life and rapid turnover rates. Minibayeva and Beckett (2001) and Beckett et al. (2003) suggested that the oxidation of epinephrine to adrenochrome can be used to indicate extracellular $O_2^{\bullet-}$ production in lichens. However, further work showed that the presence of tyrosinases (which can metabolize epinephrine) complicates this assay (Laufer et al. 2006, Zavarzina and Zavarzin 2006). Recent approaches to quantifying ROS production with fluorescent dyes are probably more specific. For example, Weissman et al. (2005a) used the probes dichlorofluorescein diacetate (DCFH-DA) and 4,5-diaminofluorescein diacetate (DAF-2DA) to study the effects of desiccation in the lichen *Ramalina lacera*. DCFH-DA is a nonpolar, nonfluorescent compound that readily diffuses across membranes. Within the cell, it is hydrolyzed by esterases to the polar, nonfluorescent, membrane-impermeable derivative DCFH. DCFH is rapidly oxidized by ROS to the highly fluorescent DCF. DAF-2DA is a nonfluorescent compound that can permeate readily into the cells, where it is hydrolyzed by intracellular esterases to generate DAF-2, which interacts with nitric oxide to form the fluorescent triazole derivative DAF-2T. Visualization of these compounds is greatly helped by using laser-scanning confocal microscopy. Experiments using DCFH-DA and DAF-2DA clearly showed that desiccation followed by rehydration increases overall ROS production in both symbionts, and specifically NO in the mycobiont.

4.4.2 *Prevention of Reactive Oxygen Species Formation*

In mitochondria, stress can disrupt normal function and increase superoxide production. Controlled uncoupling of electron flow in mitochondrial membranes from phosphorylation protects cells by reducing the formation of harmful ROS (Skulachev 1998). Uncoupling can occur, first, via the alternative oxidase (AOx) that dissipates the redox potential, and, second, via uncoupling proteins (UCP) that dissipate the proton motive force (for reviews, see Jarmuszkiewicz 2001, Hourton-Cabassa et al. 2004, Borecký and Vercesi 2005). Fungi also contain rotenone insensitive external and internal NAD(P)H dehydrogenases ("class 2"), which appear to be lower efficiency alternatives to complex 1 (Joseph-Horne et al. 2001). All these processes will result in heat

formation, and this can be monitored using microcalorimetry. Beckett et al. (2005b) used this method to measure heat production in *Peltigera polydactylon* (Fig. 4.1). Freshly collected lichens produced heat at rates of approximately $2 \text{ mW} \cdot \text{g}^{-1}$ dry mass. After desiccation for approximately 2.5 hours, heat production dropped to almost zero. Following rehydration, heat production increased to approximately $8 \text{ mW} \cdot \text{g}^{-1}$ dry mass and then gradually declined, although production was still approximately $6 \text{ mW} \cdot \text{g}^{-1}$ dry mass 3 hours after rehydration. First indications are that heat production is much higher in more desiccation-tolerant lichens (R. P. Beckett, unpublished data). These preliminary results suggest that the ability to dissipate energy may be an important component of desiccation tolerance mechanisms in lichens.

In chloroplasts, “photophosphorylation” is the mechanism used by lichen photobionts to trap the energy of the sun and make ATP and NADPH. However, as discussed, situations often exist when the light that a lichen is absorbing contains more energy than it can use to fix CO_2 . Furthermore, during recovery from stress, carbon fixation can take longer to recover than photophosphorylation (Beckett et al. 2005b). Under these conditions, the danger is that ROS can form in the photosynthetic apparatus (McKersie and Lesham 1994). Excitation energy can be transferred from chlorophyll molecules onto ground state oxygen ($^3\text{O}_2$), which is then converted into the highly toxic $^1\text{O}_2$. As discussed, $^1\text{O}_2$ can cause considerable damage; it can, for example, initiate lipid peroxidation. When high light stress is accompanied by another stress such as dehydration, the problem worsens because of further restriction of

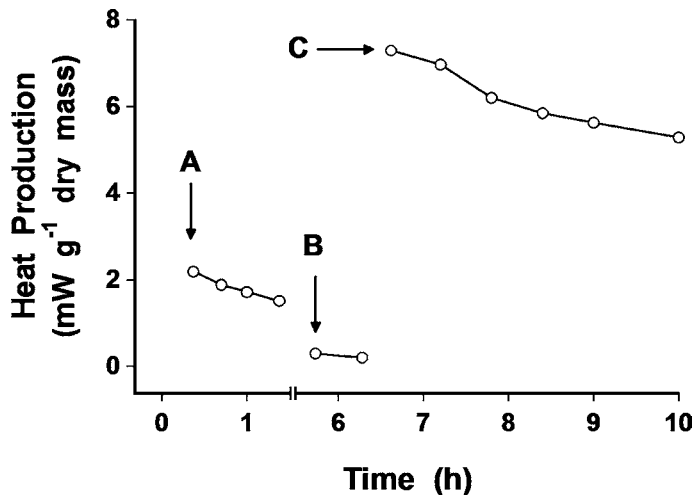


Fig. 4.1 Heat production by *Peltigera polydactylon* (A) after collection from the field, (B) after desiccation for 3 hours to a relative water content of approximately 0.1, and (C) after rehydration (modified from Beckett et al. 2005b). Error bars smaller than symbols.

photosynthesis. In higher plants, it is well known that ROS production is partly prevented by dissipating the excess energy harmlessly as heat in a process known as “non-photochemical quenching,” or NPQ (Szabo et al. 2005). To carry out NPQ, plants probably use a variety of reactions, mostly involving carotenoids. For example, beta-carotene can quench $^1\text{O}_2$. Xanthophylls, the oxygenated derivatives of carotenes, can also quench $^1\text{O}_2$, such as, lutein and neoxanthin. In particular, in the xanthophyll cycle (Frank et al. 1999, Demmig-Adams 2006), solar radiation is dissipated as heat while violaxanthin undergoes deepoxidation to antheraxanthin and then zeaxanthin, partly preventing the formation of $^1\text{O}_2$ (Fig. 4.2). With the development of readily portable chlorophyll fluorescence devices, it is now quite easy to measure NPQ in the field (Jensen 2002). Detailed analysis of seasonal variation of NPQ in the chlorophycean lichens *Lobaria pulmonaria* (MacKenzie et al. 2001, 2002) and *Xanthoria parietina* (Vráblíková et al. 2006) have shown that NPQ does indeed track the amount of solar radiation. While the relationship between desiccation tolerance and NPQ has not been rigorously investigated in lichens, NPQ in desiccation-tolerant bryophytes is higher than in desiccation-sensitive species (Deletoro et al. 1998a, 1998b). Interestingly, Catalayud et al. (1997) found that in the desiccation-tolerant lichen *Parmelia quercina*, NPQ increases as thallus water content falls. Cyanobacterial lichens often live in rather more shaded habitats than other lichens, and sometimes appear to lack NPQ (Beckett et al., 2005b). However, in some situations they can still need photoprotection. In the cyanobacterial lichen *Peltigera rufescens*, Lange et al. (1999) found good evidence that canthaxanthin formation is involved in a form of NPQ-dependent photoprotection.

Reactions involving carotenoids are the normal way that most lichens dissipate excess energy associated with high light exposure when a lichen is moist, and they even remain partially effective when desiccation occurs in the light (Kranter et al. 2005, Heber et al. 2006b). However, Shuvalov and Heber (2003) have suggested a second mechanism involving the PSII reaction center where “charge separation” results in the oxidation of P680 (the reaction center chlorophyll, a molecule associated with PSII) and, initially, in the reduction of a chlorophyll. When a lichen becomes desiccated, normal electron transfer from the reduced chlorophyll to phaeophytin is suppressed; instead, the chlorophyll may react with the protonated P680 in approximately 1 picosecond, a time frame as rapid as that proposed for zeaxanthin-dependent energy dissipation (Holt et al. 2005). Apparently, desiccation causes a change in the conformation of the PSII reaction center, such that it switches from being an energy-conserving center to an energy-dissipating center. Both mechanisms provide photoprotective pathways in green-algal lichens (Kopecky et al. 2005); in contrast, cyanolichens appear to be primarily protected by the second, desiccation activated mechanism (Heber et al. 2006a, 2006b).

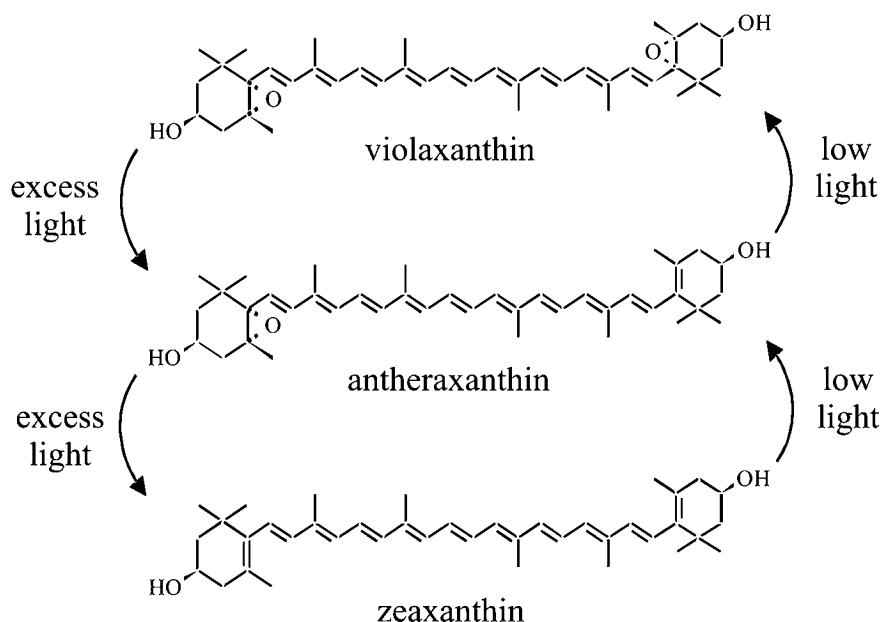
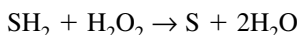


Fig. 4.2 Dissipation of excess light energy as heat in the xanthophyll cycle. In the xanthophyll cycle, violaxanthin is converted to antheraxanthin and then zeaxanthin. This pathway uses solar energy for the removal of two epoxy-groups in violaxanthin. Thus, some solar radiation is dissipated as heat rather than being involved in the excitation of chlorophyll, which can cause singlet oxygen formation due to transfer of excitation energy from chlorophyll to ground state oxygen. De-epoxidation is catalyzed by the enzyme violaxanthin deepoxidase. In higher plants, ascorbate may be involved in the reduction of the epoxy group, but which compounds contribute to this reaction in the absence of ascorbate in cryptogams is not known. Violaxanthin can be recycled by zeaxanthin epoxidase in an NADPH-dependent reaction.

4.4.3 Reactive Oxygen Species Scavenging

4.4.3.1 Enzymatic Reactive Oxygen Species Scavenging. Once formed, ROS can be removed from tissues by either enzymatic or nonenzymatic scavenging systems. Enzymes involved in scavenging cytotoxic oxygen species include superoxide dismutase (SOD), intracellular or Class I ("ascorbate") peroxidases (AP), secreted or Class II and III peroxidases, mono- and dehydroascorbate reductases, glutathione reductase (GR), and catalase (CAT) (Elstner and Osswald 1994). All aerobic organisms contain SOD, metalloproteins (the metal can be Fe, Mn or Cu and Zn) that catalyze the dismutation of $O_2^{\bullet -}$ to H_2O_2 . This will prevent the formation of the highly reactive $\bullet OH$ radical from the

reaction of $O_2^{\bullet-}$ with H_2O_2 . Peroxidases catalyze H_2O_2 -dependent oxidation of substrates (S):



Catalases break down high concentrations of H_2O_2 very rapidly but are much less effective than peroxidases at removing H_2O_2 present in low concentrations because of their low affinity (high K_m) for this substrate.



It is possible that antioxidants and enzymes actually work together to scavenge ROS, as first suggested by Foyer and Halliwell (1976). They postulated an ascorbate-glutathione cycle for the scavenging of the H_2O_2 produced from $O_2^{\bullet-}$ by SOD. This cycle involves reactions of glutathione, ascorbic acid, GR, AP, and mono- and dehydroascorbate reductases. However, it is presently unclear if lichens produce ascorbate at all—a thorough investigation of *Cladonia vulcani* showed that neither the mycobiont nor the photobiont contained ascorbate (Kranner et al. 2005). Although some fungi contain homologues of erythroascorbate (Loewus 1999), it is not clear if these erythroascorbate homologues occur in lichenized fungi and whether they can substitute for ascorbate in the above pathway.

There are few reports on the effects of desiccation on the activities of antioxidant enzymes, but the first indications suggest that no simple relationship exists between the activities these enzymes and stress tolerance (Kranner et al. 2003). Furthermore, even moderate stress appears to decrease the activity of these enzymes. For example, Mayaba and Beckett (2001) measured the activities of AP, CAT, and SOD during wetting and drying cycles in *P. polydactyla*, *Ramalina celastri*, and *Teloschistes capensis*. These species normally grow in moist, xeric, and extremely xeric microhabitats, respectively. Enzyme activity was measured shortly after collection, after rehydration in humid air for 24 hours and then wet filter paper for a further 24 hours, after desiccation for 14 days and 28 days, and during the first 30 minutes of subsequent rehydration with liquid water. In all species, enzyme activities tended to rise or remain the same following rehydration. After desiccation for 14 days, enzyme activities decreased, and then decreased further to very low values after desiccation for 28 days. In all species, including the *T. capensis* from an extremely xeric habitat, the activities of all enzymes remained at very low values during the 30 minutes following rehydration and were therefore unable to remove any ROS accumulating in lichen tissues as a result of desiccation stress. Weissman et al. (2005b) later showed that even rehydrating the desiccation-tolerant *R. lacera* collected dry from the field with liquid water

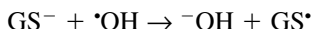
decreased SOD activity by 50% to 70% and caused a transient decrease in total catalase activities. The study of Weissman et al. (2005b) was particularly interesting, because these workers identified different SOD and CAT isoforms using gel electrophoresis. Each symbiont produced one catalase isoform. The alga contained four Fe-SOD and four Mn-SOD isoforms, while the fungus contained a Cu/Zn-SOD and a Mn-SOD. The activity of all SOD isoforms changed in a similar way in response to desiccation, although the algal CAT was more strongly inhibited than the fungal form. The apparent high sensitivity of ROS scavenging enzymes in lichens to desiccation suggests that enzymatic antioxidants are more likely to be involved in removing ROS produced during moderate stress or the normal metabolic processes of lichens rather than severe desiccation.

ROS do not only occur intracellularly. H_2O_2 can freely diffuse across the plasma membrane into the apoplast (Allan and Fluhr 1997, Henzler and Steudle 2005). Furthermore, some lichen species contain cell wall redox enzymes, such as laccases, capable of generating extracellular ROS (Laufer et al. 2006). In the apoplast, ROS can be harmful to plasma membrane- and cell wall-bound enzymes, and indeed to the plasma membrane itself. Interestingly, Beckett and Minibayeva (2007) showed that all lichens tested could rapidly breakdown exogenously supplied H_2O_2 , although most lichens apparently lack extracellular peroxidases and catalases. However, in one group of lichens, the Peltigerineae, extracellular tyrosinase activity could be readily detected, and the ability to breakdown H_2O_2 was directly correlated with tyrosinase activity. Tyrosinases have been shown to breakdown H_2O_2 using a catalase-like mechanism (Garcia-Molina et al. 2004). Peltigeralean lichens are generally more sensitive to desiccation than other lichens (Beckett et al. 2003). They are likely to produce more ROS during desiccation stress and therefore need more protection from oxidative stress. Thus, tyrosinases may defend lichens against the harmful effects of desiccation-induced ROS by breaking down H_2O_2 . It is worth noting that in addition to the ROS described above, stress can also cause the formation of other kinds of radicals, such as semiquinones (Halliwell and Gutteridge 2006). Tyrosinases are directly involved in quinone metabolism (Halaoui et al. 2006) and possibly detoxify harmful quinone radicals in lichens.

4.4.3.2 Nonenzymatic Reactive Oxygen Species Scavenging. Turning now to the nonenzymatic antioxidants, for the cells of many life forms, the major water-soluble low-molecular-weight antioxidants are glutathione (γ -glutamyl-cysteinyl-glycine [GSH]) and ascorbate (Asc) (Noctor and Foyer 1998). However, as discussed earlier, many cryptogams apparently lack ascorbate (Kranter et al. 2005, Kranter and Birtic 2005). GSH and Asc are hydrophilic, and their major function is cellular protection from oxidative damage in liquid phases, particularly in the cytoplasm. Tocopherols and beta-carotene

(Munne-Bosch and Alegre 2002) are the main lipid-soluble antioxidants and are therefore the key antioxidants in membranes (see Kranner and Birtic 2005 and Nash et al. 2006 for an overview).

Within the cytoplasm, GSH can scavenge $\cdot\text{OH}$ in the following way:



GS^\bullet , the glutathiolate anion, can react with many other molecules; in the simplest case, it reacts with another GS^\bullet , forming glutathione disulfide (GSSG):



GSSG can then be recycled by the NADPH-dependent enzyme GR:



It is difficult to overstate the importance of the ratio GSSG/2GSH. This ratio is a ubiquitous, possibly ancient, redox couple; it is a major cellular antioxidant and contributes significantly to, and appears to be representative of, the intracellular redox environment. In reviewing a large number of biological systems, Kranner et al. (2006) consider that when the redox potential of the cells of any organism rises above -160 mV, the buffering capacity of the glutathione system is lost, and macromolecules will be directly attacked by ROS. Kranner (2002) describes in detail changes in GSH metabolism during a drying-wetting cycle in desiccation-sensitive and -tolerant lichens. Initial concentrations of total GSH were similar in all species tested. In the highly desiccation-tolerant lichen *Pseudevernia furfuracea*, GSSG is normally approximately 20% of total GSH. Desiccation for 2 months increases oxidation of GSH (up to 89% GSSG), while total GSH falls by about 30% of initial values. Within 5 minutes of rehydration, GSSG was reduced back to control levels. The more desiccation-sensitive species *P. polydactyla* normally contains approximately 10% of its GSH as GSSG. After desiccation for 2 months the lichen lost about 15% of its total GSH, while the proportion of GSH as GSSG increased to 71%. However, the main difference between *Peltigera* and *Pseudevernia* was that in *Peltigera*, even after rehydration for 1 hour the proportion of GSSG only decreased to 30%. Thus, the more desiccation-tolerant species apparently recovers its normal antioxidant concentrations during rehydration more quickly than in sensitive species.

Other key enzymes concerned with GSH cycling are GR and glucose-6-phosphate dehydrogenase (6PDH). It seems likely that during the early stages of rehydration (when photosynthesis has not fully recovered), NADPH energy for the regeneration of GSH comes from the oxidative pentose shunt. The key

enzyme here is G6PDH. Comparing the activities of these enzymes during a desiccation–rehydration cycle in lichens of differing desiccation tolerance, Kranner (2002) was unable to correlate different degrees of desiccation tolerance with GR activity. However, the activity of G6PDH was dramatically decreased by long-term desiccation in the more sensitive *Lobaria pulmonaria* and *P. polydactyla* but not in the more tolerant *Pseudevenia furfuracea*. In contrast to *P. polydactyla*, *L. pulmonaria* could decrease GSSG during rehydration in liquid water as quickly as *P. furfuracea*. The difference between *Peltigera* and *Lobaria* was that in *Lobaria* G6PDH was reactivated or resynthesized extremely rapidly (within 2 minutes) during rehydration. It seems reasonable to assume that, in this lichen, the oxidative pentose shunt provided enough NADPH for rapid reduction of GSSG to GSH, which reached the level of controls after only 5 minutes. By contrast, in *P. polydactyla*, the oxidative pentose shunt probably did not provide sufficient NADPH to reduce GSSG.

Other nonenzymatic antioxidants such as Asc and tocopherol also react rapidly with ROS (Halliwell and Gutteridge 2006). It is certainly possible that lichens contain additional nonenzymatic antioxidants. Phenols have remarkable antioxidant properties in vitro (Rice-Evans et al. 1996), and many unique phenols are known in lichens (Huneck and Yoshimura 1996). However, phenolic lichen products mainly occur as extracellular crystals and hence play a limited role in metabolism so their role as cytoplasmic antioxidants is therefore uncertain. As discussed above, polyols can occur in high concentrations in lichens, and these compounds can act as antioxidants (Smirnov 1993). Lichens also contain flavonoids (Santos et al. 2000), and in higher plants, these compounds have been shown to have significant antioxidant properties. Their effects are a result of their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving ROS (Bors et al. 1990, 1994, Heim et al. 2002).

4.4.4 What Is More Important: Prevention of Reactive Oxygen Species Formation or Reactive Oxygen Species Scavenging?

While it would be convenient if desiccation tolerance were simply related to the levels of antioxidants or ROS scavenging enzymes, recent investigations have shown that no such simple correlation exists (e.g., Mayaba and Beckett 2001). Indeed, sensitive species often have higher concentrations of antioxidants (Kranner et al. 2003). However, a characteristic of well-adapted species appears to be an ability to rapidly reestablish the species-specific normal antioxidant concentrations during rehydration after desiccation. This may suggest that prevention of ROS formation during recovery may be more important than ROS scavenging.

4.5 Other Mechanisms of Desiccation Tolerance

4.5.1 Anatomical Adaptations

In lichens, water uptake, storage, and loss are predominantly controlled by physical processes, and thallus morphology and anatomy have minor effects (Rundel 1988). We still have very little idea how lichens can tolerate the huge reduction in cell volume that accompanies desiccation. Recent work on bryophytes has suggested that a key feature of desiccation tolerance is the depolymerization of the microtubule cytoskeleton (Pressel et al. 2006), and it would be interesting to test if the same thing happens in lichens. Beckett (1995) estimated that the elastic modulus of lichen cell wall is rather low, implying that during drying, walls will tend to readily contract around a shrinking protoplast. However, despite this, Honegger et al. (1996) used elegant scanning and transmission electron microscope cryotechniques to demonstrate that large cytoplasmic gas bubbles are formed within hyphae and suggested that these bubbles help the protoplast to remain in contact with the wall during drought. Upon rehydration, these gas bubbles rapidly disappear and the protoplast refills the cells. It is certainly true that although some vertical shrinkage of whole lichen thallus occurs during drying, the overall shape of most lichens remains relatively unchanged, presumably because of the rigidity of the fungal cell walls. It will be interesting in future to study the impact of gas bubble formation on the cytoskeleton of fungal cells.

4.5.2 Ability to Use Water Vapor

An important adaptation of lichens to water shortage is their ability to use not only liquid water but also water in the form of vapor, fog, and dew. For reasons that are unclear, while lichens with chlorophycean photobionts can achieve net photosynthesis from atmospheric moisture alone, species with cyanobacterial photobionts need liquid water (Lange et al. 1986). However, interestingly, desiccated cyanobacteria isolated from lichens in axenic cultures can display net photosynthesis following uptake of water vapor alone (Lange et al. 1994). Lange and his co-workers have elegantly demonstrated the remarkable ability of some desert lichens to achieve net photosynthesis with nonliquid water, for example, in the Negev Desert, Israel (Lange et al. 1970a, 1970b), in the coastal fog zones of Chile (Lange and Redon 1983), and in Namibia (Lange et al. 1990). *Ramalina maciformis* from the Negev Desert in Israel can carry out net photosynthesis when the relative humidity exceeds 80%, and at a relative humidity of 95%, CO₂ uptake is identical to that of a fully hydrated thallus. In *Teloschistes capensis* growing in the coastal fog zone of the Namib desert, heavy dewfall, supplemented by fog, typically increases

the thallus water to 60% by midnight. A small but significant amount of respiration occurs during the night. After sunrise, lichens can achieve net photosynthetic rates of up to $16 \text{ nmol CO}_2 \text{ g}^{-1} \cdot \text{sec}^{-1}$ for a few hours until thalli dry out, and photosynthesis and respiration stop (Fig. 4.3).

In very cold habitats such as mainland Antarctica, the metabolic activity of lichens is severely limited by water availability and low temperatures. Remarkably, studies (e.g., Schroeter and Scheidegger 1995) have shown that desiccated lichens can take up enough water to photosynthesize from the sublimation (vapor phase) of snow. The lowest temperature measured for net photosynthesis was -17°C for *Umbilicaria aprina* at a continental Antarctic site.

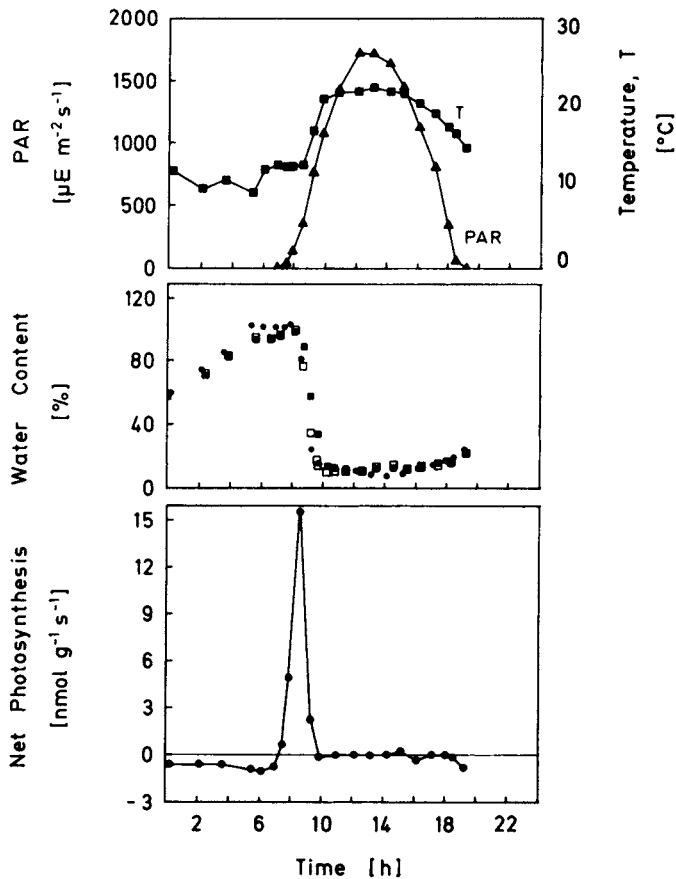


Fig. 4.3 Daily time course of microclimatic parameters and responses of *Teloschistes capensis* in the coastal fog zone of the Namib desert. **Top**, air temperature and photosynthetically available radiation (PAR). **Center**, water content of replicate samples of lichens (different symbols). **Bottom**, CO_2 exchange per unit dry weight (CO_2 uptake positive). Modified from Lange et al. (1990).

Even if lichens are “hydrated,” at low temperatures they will be subjected to severe “physiological drought.” For example, if water-saturated thalli of *U. aprina* were slowly cooled at subzero temperatures, ice nucleation activity could be detected at -5°C , indicating extracellular freezing of water. Extracellular ice formation leads to cytorrhysis (cell collapse) of the photobiont cells and cavitation of the mycobiont cells. However, both processes were reversible if the lichen thallus was rewarmed. Furthermore, even if lichens were frozen in a hydrated state, they could still photosynthesize at subzero temperatures. Later studies in Antarctica (Pannewitz et al. 2003) showed that although the snow cover represented the major water supply, lichens became active only for a short period when the snow melted. The snow did not provide a protected environment, as occurs in some alpine habitats, but appeared to limit lichen activity.

4.5.3 Biochemical Adaptations

Apart from reducing ROS formation, results obtained from other organisms would suggest that lichens could tolerate desiccation using a combination of sugars, amphiphilic substances, and dehydrins. Nonreducing sugars such as trehalose and sucrose are thought to promote vitrification (i.e., the formation of a “glass phase” in the cytoplasm). Vitrification is a phenomenon that has been studied extensively in the “orthodox” (i.e., desiccation-tolerant) seeds (Black and Pritchard 2002). In the glassy state, the cytoplasm has the properties of a liquid with the viscosity of a solid. The ability of the cytoplasm of quiescent seeds to vitrify confers many benefits. Due to the high viscosity of the cytoplasm, possibly harmful chemical reactions proceed much more slowly, and alterations in ionic strength and pH and solute crystallization are prevented. The glass phase also fills space, thus preventing cellular collapse following desiccation. Additionally, nonreducing sugars may substitute for water by forming hydrogen bonds, maintaining hydrophilic structures in their hydrated orientation (Crowe et al. 1984), and thus helping to stabilize protein structure and membranes under dry conditions (for a review, see Leprince et al. 1993). Polyols (polyhydric alcohols) such as sorbitol and mannitol also accumulate in plants during water deficit (Popp and Smirnov 1995). Whether sugars and polyols play a role in desiccation tolerance in lichens is unknown, but certainly lichens contain high concentrations of these compounds (Roser et al. 1992). Interestingly, Beckett (1995) found that the proportion of the cytoplasmic osmotic potential attributable to ions (mostly K^+) varies from about 10% in desiccation-tolerant species to 75% in highly sensitive species. The implication is that sugars take over the role of potassium in generating turgor (needed for cell division, for example) in more tolerant species and therefore can contribute to the protection of lichens from the effects of desiccation.

In seeds, pollen, and resurrection higher plants, “amphiphilic substances,” substances with some solubility in water and lipids, are believed to play an important role in desiccation tolerance (Oliver et al. 2001, 2002). Many types of amphiphilic substances exist in plants, including alkaloids, flavonoids, and other phenolic compounds. Interestingly, the distribution of these compounds within the plant cell differs depending on hydration level, with the amphiphiles relocating from the aqueous cytoplasm to the membrane bilayer as water is removed. Here, they can act as powerful antioxidants, and strongly stabilize membranes. It will be interesting to see if future research reveals the presence of amphiphiles in lichens.

Dehydrins are a type of late embryogenesis abundant (LEA) proteins, which are characterized by high glycine content and high hydrophilicity index. As their name suggests, they are abundant in many seeds (Rorat 2006) but also occur in many bacteria and fungi (Abbá et al. 2006). From their amino acid sequences, it has been predicted that some classes of LEA proteins exist as random coil structures and others as amphipathic alpha helical structures. In comparison with a pure sucrose glass, the presence of LEA proteins increases both the glass transition temperature and the average strength of hydrogen bonding of the amorphous sugar matrix. LEA proteins could play a structural role as anchors in a tight molecular network to provide stability to macromolecular and cellular structures in the cytoplasm in the dry state. In the highly viscous or vitreous cytoplasmic matrix, this network would inhibit fusion of cellular membranes, denaturation of cytoplasmic proteins, and effects of harmful free radical reactions. It is worth noting that dehydrins have been suggested to play various other roles in the tolerance of organisms to stresses other than desiccation (Wise and Tunnacliffe 2004). Very preliminary evidence suggests that dehydrins may play a role in desiccation tolerance of the lichen *Peltigera horizontalis* (Schulz 1995), but much more work is needed to assess the role of dehydrins in the desiccation tolerance of lichens.

4.6 Constitutive and Inducible Desiccation Tolerance

If tolerance increases as a result of exposure to prior stress, the plant is said to be acclimated (or hardened). In theory, both “protection” and an “ability to repair” could be induced. It is often assumed that lichens rely mainly on constitutive mechanisms, as they frequently grow in highly stressful environments where stresses such as desiccation may be sudden and severe. In this respect, they may differ from higher plants. A lichen exposed to dry air will dry to the point where metabolism ceases within a few minutes, while a drying shoot of a vascular resurrection plant may remain metabolically active for many hours. If the lichen is to survive, its desiccation tolerance *must* be constitutive; the

vascular plant has time to put a protective mechanism in place when drought threatens. However, the disadvantage of constitutive mechanisms is that they are present even when not needed, and at these times they divert energy away from growth and reproduction. Selection may therefore favor inducible tolerance mechanisms in environments that are usually moist and in which lichens are predictably (and probably slowly) desiccated. The latter conditions are typical for the habitats where many cyanobacterial lichens grow. Although we have known for some time that lichens can acclimatize their photosynthetic and respiratory apparatus to work optimally under changing environmental conditions (Kershaw 1984, Lange and Green 2005), work on the acclimation of lichens to environmental stresses is just beginning. Recently, Beckett et al. (2005b) successfully hardened the lichen *Peltigera polydactylon* to desiccation stress by slowly dehydrating thalli to a relative water content of approximately 0.65 for 3 days and then storing them fully hydrated for an additional day. This treatment significantly improved the ability of thalli to recover net photosynthesis during rehydration after desiccation for 15 but not 30 days (Fig. 4.4). Interestingly, pre-treating thalli with the stress hormone abscisic acid (ABA) could substitute for partial dehydration, suggesting that ABA is involved in signal transduction pathways that increase tolerance. The mechanisms involved in this increase are unknown, but one possibility, known from free-living cyanobacteria (Potts 1994) could be the synthesis of O₂ binding protein that would reduce ROS formation. Heber et al. (2006b) also suggest that drying lichens with chlorophycean photobionts in the light rather than the dark can increase their NPQ during subsequent rehydration, indicating that light can be a signal for hardening. The implication of these results is that hardening may be important even for lichens growing in habitats where they experience rapid desiccation, but more work is needed on the balance between constitutive and inducible tolerance mechanisms in lichens.

4.7 Desiccation Tolerance: Protection or Repair?

From the foregoing discussion, it is clear that we are still far from understanding fully the mechanisms of desiccation tolerance in lichens. One of the fundamental questions is whether desiccation tolerance is largely a matter of reassembly and reactivation of components conserved intact through a time of stress or, alternatively, an extensive “repair” process is involved. During rehydration following desiccation some parameters, such as chlorophyll fluorescence, recover almost immediately following rehydration (e.g., Beckett et al. 2005b). This suggests that the integrity of the thylakoids is preserved throughout the events of desiccation and remoistening. On the other hand, the fact that *complete* recovery of carbon fixation can take much longer suggests the involvement in

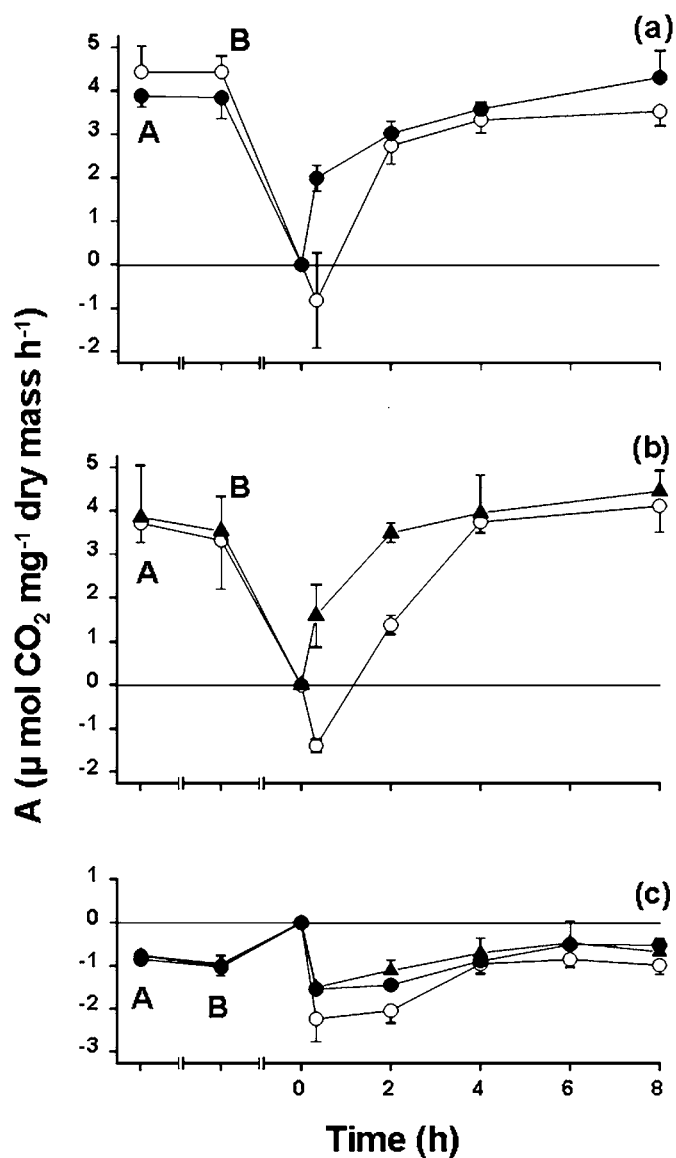


Fig. 4.4 Both partial dehydration (a) and treatment with 100 μM ABA (b) improved the recovery of photosynthesis during rehydration following desiccation for 15 days in *Peltigera polydactylon*. Neither pretreatment had a significant effect on respiration (c). A = after collection from field, B = following 3 d pretreatment. Time 0 indicates the start of rehydration. *Open circles*: control material; *solid circles*: material pretreated by partial dehydration; *solid triangles*: ABA pretreated material. Modified from Beckett et al. (2005b).

recovery of other cellular mechanisms, as yet unknown. Furthermore, during rehydration following desiccation, membranes are initially leaky to ions and metabolites but later regain their integrity (Weismann et al. 2005a). Therefore, it is possible that some form of repair-based desiccation tolerance mechanisms exists. In bryophytes, initial studies on moss ultrastructure during and after desiccation seemed to imply that some form of repair takes place. Freshly rehydrated plants appear to display swelling of chloroplasts and mitochondria and major changes in the endomembrane domains and microtubular cytoskeleton, damage that is repaired gradually. However, recent more careful investigations using improved procedures for preparing material for microscopy (e.g., Pressel et al. 2006, Proctor et al. 2006) no longer support a simple damage–repair hypothesis of desiccation tolerance. The latest view is that tolerance involves a suite of protective mechanisms, including scavenging ROS or preventing their formation, and probably synthesizing sugars and dehydrins. In bryophytes, recovery of the essential systems, responsible for respiration, light-capture and CO₂ fixation, and protein synthesis, now looks to be largely physical and probably not metabolically costly in terms of either energy or materials. We need to do more work to test if the same is true for lichens. If so, then the “cost” of a lichen being so stress tolerant may well be mainly in producing “protective mechanisms” that enable lichens to survive the next stress event.

4.8 The Evolution of Desiccation Tolerance in Lichens

The development of desiccation tolerance has played a major role in the evolution of lichens. The classic study of Lutzoni et al. (2001) showed that there were regular but infrequent gains of lichenization during Ascomycota evolution, but multiple losses, and that major lineages in the Ascomycota are derived from lichen-forming ancestors. The following section reviews a case study on the evolution of stress tolerance in the lichen *Cladonia vulcani*. Kranner and Lutzoni (1999) hypothesized that lichenization would expose both lichen symbionts to additional oxidative stress, and this idea was developed by Kranner et al. (2005). Lichenization requires that the fungus gives up its saprophytic lifestyle below ground; the photobiont may also cease its hidden life in bark, soil (Mukhtar et al. 1994), or small crevices in rocks (Ascaso et al. 1995). By contrast, a lichen grows on the surface of its substratum and is a complex structure that neither a fungus nor an alga can form alone. As a result, both symbionts are exposed to much higher levels of solar radiation and desiccation and, as a consequence, oxidative stress. Kranner et al. (2005) conducted a detailed investigation into the mechanisms involved in protection from oxidative stress in the intact lichen *Cladonia vulcani* and in its symbionts when isolated and grown in axenic culture. This study showed that antioxidant

and photoprotective mechanisms in the lichen *Cladonia vulcani* are more effective by two orders of magnitude than those of its isolated partners. The major low-molecular-weight antioxidant in the fungus of this species is GSH. Neither the *Cladonia vulcani* mycobiont nor the photobiont contained Asc; tocopherol was found only in the photobiont. In isolation, both alga and fungus sustained oxidative damage during desiccation: on its own, the alga tolerated only very dim light and its photoprotective systems were only partially effective. Without the alga, the fungus's GSH-based antioxidant system was slow and ineffective. However, in the lichen, the two symbionts appeared to induce upregulation of protective systems in each other. In the lichen, where it was exposed to higher light intensities, the alga had lower chlorophyll concentrations, which helped to avoid $^1\text{O}_2$ formation. In addition, it contained higher concentrations of photoprotective pigments involved in NPQ and of the antioxidant alpha-tocopherol. In addition, total glutathione (GSH plus GSSG) was present in the lichen at a level 30% greater than the sum of the contents in isolated alga and fungus. The lichen therefore appeared to be able to cope more successfully with oxidative stress than its isolated partners. The authors noted that for this lichen, the mutually enhanced resistance of its symbionts to oxidative stress, and in particular the lichen's enhanced desiccation tolerance, are required for life above ground with the major benefit of the new lifestyle being the increased chance of dispersal of reproductive propagules. Overall, the enhanced antioxidant capacity apparently contributes to the evolutionary success of the lichen symbiosis (Kranter et al. 2005).

4.9 Conclusions

According to the "competitor-ruderal-stress tolerator" model of plant life histories proposed by Grime (1979), lichens would be classified as classic stress tolerators. High-stress habitats should favor species with inherently low growth rates, and indeed many lichens grow very slowly (see Chapter 9 for more details). Slow growth is probably a result of first low resource availability (mostly water, which limits the time lichens can be active), and second metabolic "costs" associated with being desiccation tolerant. It is not always easy to know what these metabolic costs are. One cost is "resaturation respiration," a substantial elevated CO_2 release that occurs during rehydration following desiccation (Nash et al. 2006). Other costs are more subtle. For example, in mosses, an increase in NPQ associated with hardening to desiccation tolerance can reduce photosynthetic efficiency (Beckett et al. 2005a). Similarly, mitochondria with strong AOX or UCP activity will respire less efficiently. The need to synthesize sugars, polyols or dehydrins to protect cells during desiccation will divert reserves away from growth and respiration.

Despite their undoubted “inefficiency,” it is their extraordinarily high desiccation tolerance that enables them to live in places that no other plants can.

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5 Desiccation Tolerance: Gene Expression, Pathways, and Regulation of Gene Expression

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5.1 Introduction

Desiccation tolerance means that plant tissues have the ability to become air-dry without loss of viability and successfully rehydrate when water becomes available. *Desiccation* is a severe water loss, and it has to be distinguished from *dehydration*, which is a mild water stress. Most plants can survive dehydration and tolerate a water loss up to 20% or even 30% of their initial water contents. Desiccation tolerance in higher plants is rare, and it is found in vegetative tissues of a unique group of plants termed *resurrection plants* and in seeds of most higher plants. Desiccation tolerance in seeds is strictly developmentally controlled; it is acquired during seed formation but it is lost during early stages of germination. It is estimated that around 250 to 300 species of desiccation-tolerant resurrection plants exist. They occur in specialized ecological niches with temporally restricted water availability, mainly in the southern hemisphere (Porembski and Barthlott 2000, Bartels et al. 2006). Phylogenetic analyses indicate that desiccation tolerance in land plants is a basal trait (Alpert 2005).

This chapter treats the question, What is known about the genomic components required to render plant tissues desiccation tolerant? Genomic components include DNA sequence information of nuclear and organelle genomes, the processes and mechanisms of genome expression, and signaling mediating gene expression. Up to now, complete or extensive genome sequences are available from *Arabidopsis thaliana*, rice, maize, *Medicago truncatula*, and poplar but not from any higher plants with desiccation tolerance in vegetative tissues. Therefore, a broad genomic analysis is not yet possible. The best-studied desiccation-tolerant plants in this respect are the resurrection plant *Craterostigma plantagineum* (Bartels and Salamini 2001), *Xerophyta villosa* (Collett et al. 2004), and *Sporobolus stapfianus* (Neale et al. 2000). Data available from desiccation-tolerant plants will be compared with data from *Arabidopsis thaliana*, the genetic model plant that expresses desiccation tolerance in seeds. In addition to gene expression programs in seeds, dehydration stress has been extensively studied in vegetative tissues of *A. thaliana*, and we review some of those data as far as we believe that they are relevant to the understanding of desiccation.

It is hard to reach unifying mechanisms that determine desiccation tolerance in plants. The reasons for this are partly due to the complex aspects of plant responses to water depletion. There is no single mechanism or control scheme for plant desiccation tolerance, since it is a multifactorial mechanism, which is not understood and relies on correlations between readjustment of metabolic, cellular, and genetic components. Desiccation tolerance may depend mainly on changes in gene expression. Most, if not all plants, express the genes necessary for desiccation tolerance in seeds or pollen grains, but the desiccation tolerance program is under developmental control and not fully expressed in vegetative tissues. The analysis of regulatory switches may provide clues for understanding the necessary parameters of desiccation tolerance. Therefore, comparisons of genes associated with desiccation tolerance should be analysed on two levels: structural genes and regulatory genes. Some of the genes and even mechanisms involved in desiccation tolerance also seemed to be expressed in nontolerant plants in response to dehydration, although to a lesser extent.

5.2 Whole Plant Effects: Growth and Metabolism

Before discussing which genes may be major players in desiccation tolerance, we will first summarize some features of whole plants when subjected to desiccation; this may help to link gene expression to physiology, which is necessary to understand the phenomenon of desiccation tolerance.

One adaptive feature in response to water deficit is an inhibition of growth and metabolism, especially photosynthesis and respiration. The extent of drought tolerance is often inversely related to growth rate. For example, leaf elongation rates are reduced by salt and water stress, although transiently, with persistently reduced leaf growth rates with more extreme changes in salinity (Munns et al. 2000). Although water stress inhibits the growth of aerial plant organs, roots may continue to elongate (Spollen et al. 1993), as an adaptive mechanism enabling water uptake from deeper soil layers. A major cause of reduced growth rate is a decreased CO₂ assimilation rate due to reduced stomatal conductance and activities of photosynthetic enzymes including RUBISCO (Ramachandra et al. 2004). Photosynthesis is usually affected when relative water content falls below 50% to 70% and also due to effects on cell and chloroplast shrinkage and crystallization of enzymes (Kaiser 1987). One adaptive feature of resurrection plants such as *Xerophyta humilis* is the ability to rapidly and reversibly shut down photosynthetic genes during dehydration and induce their expression rapidly following rehydration (Collett et al. 2003). The constitutive overexpression of several stress-related genes such as *CBF1*, *DREBD1A*, and *ATHB7* and yeast trehalose synthase has been shown to cause slower growth (Holström et al. 1996).

Both cell division and cell expansion are coordinately regulated in plants and are affected by water availability. Water stress was shown to inhibit transcript levels of the cyclin-dependent kinase *ZmCdc2* in maize, decreasing the rate of cell division (Setter and Flannigan 2001). Cell expansion necessarily involves acid-induced cell wall loosening, which is caused by expansins (Cosgrove 1997). In maize, transcription of three expansin genes, *Exp1*, *Exp5*, and *Exp8*, was upregulated in the apical region of roots, which correlated with root elongation at low water potential (Wu et al. 2001). Expansins have also been shown to be implicated in the increases in cell wall flexibility observed during cell shrinkage and rehydration in the desiccation-tolerant plant *C. plantagineum*: the expansin gene *CpExp1* was closely correlated with observed changes in expansin activity during leaf dehydration and rehydration (Jones and McQueen-Mason 2004).

5.2.1 Desiccation-Induced Changes in Membranes and Subcellular Organelles

Few studies have been performed on ultrastructural changes in higher plants occurring during desiccation. Most descriptive studies have been performed on drought-tolerant plants such as resurrection plants, which are efficient in withstanding drought via adaptive ultrastructural changes. For *C. plantagineum*, desiccation causes the cytoplasm to shrink, forming an open network containing the organelles. Polyribosomes are still present although the chloroplasts become oval and lose some internal organization (Schneider et al. 1993). During slow dehydration in the resurrection plant *Craterostigma wilmsii*, 60% of the chlorophyll and 20% of carotenoids are lost (Cooper and Farrant 2002). Mitochondria of desiccation-tolerant plants are usually swollen after drying, with indistinct cristae (Bewley and Pacey 1978). Often, conspicuous cell wall folding occurs and fragmentation of the vacuole (Cooper and Farrant 2002). Recent studies on membrane composition in the resurrection plant *Ramonda serbica* show a significant reduction in plasma membrane lipid content during drying, with a high level of free sterols and fewer phospholipids and a decrease in the unsaturation of the total lipid fraction (Quartacci et al. 2002). Vitré et al. (2004) report ultrastructural and biochemical changes in the cell wall of *C. wilmsii* on desiccation, including cell wall folding and a decrease in polysaccharide content.

Membranes are sensitive to changes in water concentration. To maintain membrane bilayers in a liquid-crystalline phase when water is removed is a major control point in desiccation tolerance, to keep them fluid and prevent them entering the gel phase. Sugars, particularly sucrose, lactose, and raffinose, are effective in replacing membrane water and maintaining the hydrophobic-hydrophilic orientation of the membrane phospholipids, although sugars vary in their membrane stabilizing efficiency (Crowe and

Crowe 1986). The best stabilization model is one whereby three hydrogen bond bridges exist from three sugar hydroxyl groups and two nonesterified oxygens of one phosphate head group and one oxygen of an adjacent phospholipid molecule (Gaber et al. 1986).

5.2.2 *Cytological Changes*

Extreme desiccation causes crystallization of cytoplasmic proteins and solutes, which may result in cell injury (Leopold 1990). Work on cellular changes upon desiccation have been mainly restricted to cells in developing seeds, which must acquire desiccation tolerance as a physiological development step in the maturation process. However, many of these control mechanisms may be more widely relevant in vegetative desiccation tolerance. Vitrification or glass formation is a general mechanism to avoid the crystallization of cellular components upon drying. A glass is a supersaturated liquid solution with the viscosity of a solid, and its formation involves no chemical or physical change in the solution (Burke 1986, Williams and Leopold 1989). This state will prevent freezing and will readily melt into a liquid phase following the addition of water, without cellular injury (Bruni and Leopold 1991). As the cytoplasm dries to below $0.3 \text{ g H}_2\text{O} \cdot \text{g dry weight}^{-1}$, the molecular mobility of the cytoplasm firstly decreases by more than five orders of magnitude (Buitink et al. 1999) and the cytoplasm vitrifies at about $0.1 \text{ g H}_2\text{O} \cdot \text{g dry weight}^{-1}$. The other adaptive advantages of the glass state are that due to its high viscosity, diffusion is impeded, so that degradative processes such as those caused by reactive oxygen species (ROS) are inhibited, and solutes which would otherwise become concentrated are trapped, thereby buffering against osmotic and pH changes (Leopold 1990, Koster 1991). Additionally, the glass phase effectively ensures that cell space is filled so that water loss does not result in cell collapse and macromolecular structure is maintained via hydrogen bonds between water in the glass state and macromolecules. Although these mechanisms cannot account for desiccation tolerance per se, they function to stabilize structures and cellular components during the drying process. Glass formation is promoted by sugars at ambient temperatures as water is removed from seeds (Koster 1991). Data suggest that during drying of desiccation-sensitive tissues, a de-esterification of the acyl chains of the glycerol component of phospholipids occurs (Senaratna et al. 1987).

At physiological hydration, the phospholipid bilayer is in a liquid-crystalline phase, which permits rotational and lateral movement of phospholipids and integral proteins within the bilayer. Water loss may result in membrane phospholipids entering a gel phase (Hoekstra et al. 1991), where the packing of lipid acyl chains is tighter, resulting in restricted rotational and lateral mobility of the lipids affecting membrane permeability. Because a gel phase for

membranes is associated with non-desiccation-tolerant cells, as the associated degree of membrane reorganization allows cytoplasmic solute leakage and disruption of membrane enzyme complexes from which cells cannot recover, the maintenance of a liquid-crystalline state is a prerequisite for controlling desiccation and salt tolerance. The membrane phospholipid phosphatidyl ethanolamine (PE) is more dependent on the dehydration state than other membrane phospholipids, when the water content falls below two water molecules per PE molecule, it will readily form a hexagonal phase, which completely disrupts the membrane bilayer. Under water stress, there is a general decrease in the membrane lipid components (Monteiro de Paula et al. 1990), which can be correlated with an inhibition of lipid biosynthesis (Monteiro de Paula et al. 1993) and a stimulation of lipolytic and peroxidative activity (Ferrari-Iliou et al. 1994, Sahseh et al. 1998).

5.3 Control of Water Permeability: The Role of Aquaporins

One control point of dehydration in plants is at the level of facilitated water permeability. Water transport through vegetative tissues occurs via transcellular and intercellular pathways (Chrispeels and Maurel 1994, Maurel 1997). Major intrinsic proteins (MIPs) or aquaporins facilitate the diffusion of water, glycerol, and small organic molecules or gas transfer through membranes, thereby serving as water channels and having a role in water homeostasis (see review in Kjellbom et al. 1999). Plants have more aquaporin genes than species from other kingdoms, and these are believed to encode central components in plant-water relations (Tyerman et al. 2002). In plants, aquaporins may be assigned into four major groups, based on sequence similarity (Tyerman et al. 2002). They may be also defined by their subcellular localization (Maurel 1997) either being located at the vacuolar membrane (tonoplast intrinsic proteins [TIPs]) or at the plasma membrane (plasma membrane intrinsic proteins [PIPs]). A further class, not associated with cellular structures, includes rMPI from rice (Liu et al. 1994) and NLM1 from *Arabidopsis* (Weig et al. 1997). Enhanced PIP and TIP aquaporin expression has been observed in the desiccation-tolerant plant *C. plantagineum* (Mariaux et al. 1998), consistent with a regulatory role in maintaining cell turgor. In another desiccation-tolerant plant, *Sporobolus stapfianus*, high-level expression of a γ TIP is confined to desiccation-tolerant tissue, which suggests that the protein function may be required for the cells to accommodate the large differences in osmotic potential during dehydration and rehydration, and it may facilitate the rehydration process (Neale et al. 2000). Participation of a TIP in water fluxes in desiccation-tolerant plants is also supported by data on the resurrection grass *Eragrostis nindens* (Vander Willigen et al. 2004). In spinach plasma

membranes, aquaporins comprise as much as 20% of the total integral plasma membrane protein (Johansson et al. 1996). Antisense studies in tobacco showed that impaired NtAQP1 expression (a PIP plasma membrane aquaporin) resulted in lower root hydraulic conductivity and decreased water stress resistance (Siefritz et al. 2002). However, drought stress has also been shown to downregulate transcripts of three aquaporins in *Nicotiana glauca* (Smart et al. 2001), presumably resulting in decreased membrane permeability, to perhaps facilitate water conservation during dehydration stress. For the aquaporin PM28A, it has been shown that phosphorylation and dephosphorylation regulate water transport activity in vivo (Johansson et al. 1998). A consensus phosphorylation site is conserved for all PIP2 plasma membrane aquaporins (Kjellbom et al. 1999). Aquaporins may be regulated by plant hormones like abscisic acid (ABA) (Kaldenhoff et al. 1993) or gibberellins (Phillips and Huttly 1994), and it is additionally thought that gating behavior is controlled by pH and pCa^{2+} (Tyerman et al. 2002). Aquaporin expression is highly correlated with cells associated with large fluxes of water, and a model for their role in the cytosolic osmoregulation at the single-cell level involves differential regulation of plasma and vacuolar membrane aquaporins via phosphorylation and dephosphorylation to control water flux (Kjellbom et al. 1999).

5.4 Sensing of Water Deficit

It is not certain how osmotic stress is sensed in plants, and there are no data from desiccation-tolerant plants. Given the similarity of the gene expression program between sensitive and tolerant plants during the initial phase of dehydration, sensors for water loss may be identical. Sensing of water stress may be via stretch-activated channels and cell wall-associated kinases (Kohorn et al. 2001). The hyperosmotic stress-sensing pathway in yeast is, however, better characterized, probably consisting of the SLN1 histidine kinase two-component regulatory system, which feeds into the HOG mitogen-activated protein kinase (MAPK) kinase pathway (Reiser et al. 2003). In *Arabidopsis*, several candidates for osmosensors exist, together with circumstantial evidence that a similar mechanism to that in yeast may operate. An *Arabidopsis* putative homologue of yeast SLN1, ATHK1 was isolated, which is upregulated by changes in osmolarity and is a functional histidine kinase (Urao et al. 1999). ATHK1 can complement yeast *sln* deficient mutants and therefore acts as an osmosensor in yeast, and by analogy, it is suggested that this mechanism is conserved in higher plants. A further possible higher plant osmosensor candidate is NtC7, a receptor-like membrane protein whose transcript is induced by wounding, drought, and salt stress in tobacco (Tamura et al. 2003). Overexpression of NtC7 conferred increased tolerance to mannitol osmotic

stress but not NaCl stress, suggesting a function in osmotic sensing independent to ion homeostasis. Yet a further osmosensor candidate is cytokinin response 1 (Cre1), a putative cytokinin sensor in *Arabidopsis*. Cre1 is also a histidine kinase that functions in a two-component signaling cascade and is regulated identically by turgor pressure and by cytokinins (Reiser et al. 2003).

5.5 Signal Transduction: Second Messengers and Signaling Molecules

Complex cascades of events link the perception of osmotic stress to osmoregulatory changes in gene expression (Fig. 5.1). These include ABA accumulation, redistribution of intercellular Ca^{2+} , phospholipid signaling, ROS, and protein phosphorylation. Calcium functions as a second messenger in plants

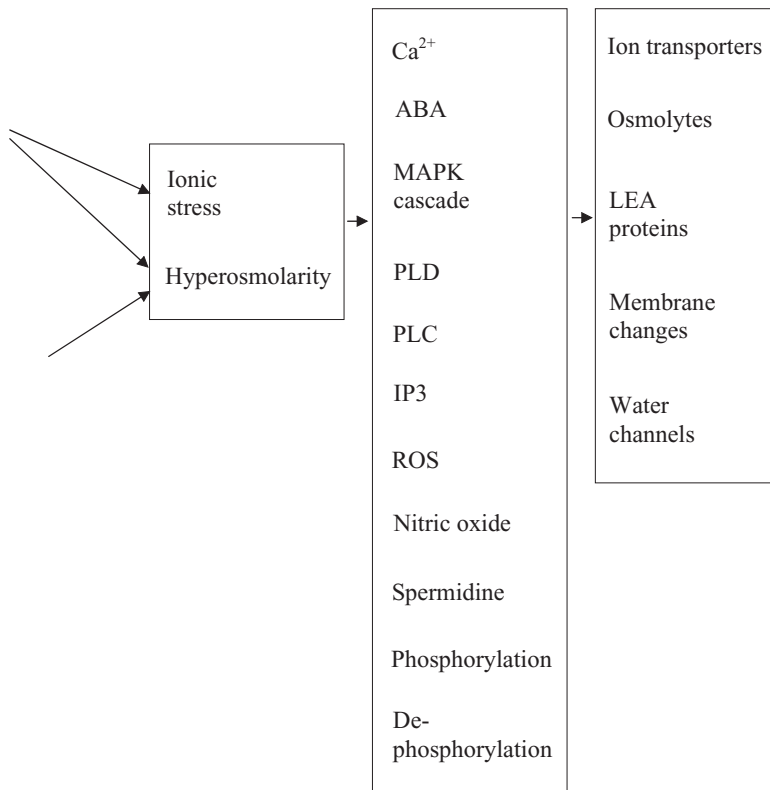


Fig. 5.1 A generalized scheme summarizing the resulting aspects of salt and drought stress following sensing, namely, ionic stress and hyperosmolarity and various identified signalling molecules that translate pathways into downstream adaptive responses.

to couple extracellular stimuli to intercellular responses (Snedden and Fromm 1998; 2001). There are three major classes of Ca^{2+} sensors that have been characterized in plants: calmodulin (CaM) and CaM-related proteins (Snedden and Fromm 1998), calcineurin B-like (CBL) proteins (Kudla et al. 1999), and calcium-dependent protein kinases (CDPKs) (Harmon et al. 2000). CaM and CBL are small proteins having multiple Ca^{2+} binding sites that relay the Ca^{2+} signal by interacting with target proteins, forming a complex signaling network of interconnected pathways (Luan et al. 2002). A rapid increase in free cytosolic calcium was observed following drought and salt treatments in intact whole *Arabidopsis* plants (Knight et al. 1997), although it is now known that different specific calcium signatures (the induced concentration of Ca^{2+} , its transiency, and kinetics of induction) result from different stimuli (Trewavas 1999, Reutel and Knight 2004), depending on the cell type (Kiegle et al. 2000). Some examples of calcium binding proteins involved in drought or salt stress responses are the *Arabidopsis CBL1* gene whose transcription is regulated by salt and drought (Kudla et al. 1999) and whose mutation results in impaired tolerance to these stresses (Albrecht et al. 2003). The expression of a CaM-binding protein AtCAMBP25 in *Arabidopsis* was also shown to be correlated by antisense or overexpression studies with increased sensitivity or tolerance, respectively, to ionic and nonionic osmotic stress (Perruc et al. 2004). A further group of six Ca^{2+} -binding proteins in *Arabidopsis* was shown to be variously induced by multiple environmental stresses, including salt stress, and therefore probably involved in multiple stress signal transduction pathways (Yang and Poovaiah 2002).

5.5.1 Protein Phosphorylation and Dephosphorylation

Protein phosphorylation and dephosphorylation are general mechanisms of signal integration and regulation of many pathways in plants. The mitogen-activated protein kinase (MAPK) cascades are common signaling pathways in plants as well as in animals. In *Arabidopsis*, MAPK, MAPKK, and MAPKKK genes comprise large gene families (Riechmann et al. 2000). MAPK pathways are involved in osmotic stress response signaling pathways (Munnik and Meijer 2001), and it has been shown that several of these MAPK genes may be induced by multiple independent stresses (Knight and Knight 2001) to allow cross-talk between stress pathways. Examples of kinases specific to drought- and salt-tolerance pathways are McCDPK1, a salinity- and drought-induced calcium-dependent protein kinase from *Mesembryanthemum crystallinum* (Partharkar and Cushman 2000), and the p44MMK4 kinase, activated by drought in alfalfa (Jonak et al. 1996). In addition, two *Arabidopsis* calcium-dependent kinase genes (*cATCDPK1* and *cATCDPK2*)

are upregulated by drought and salt stress (Urao et al. 1994), and in wheat, a serine-threonine-type protein kinase accumulates in dehydrated shoots (Anderberg and Walker-Simmons 1992). There is evidence that plant cells have different osmosensing pathways that are able to distinguish between moderate and extreme osmotic stress (Munnik et al. 1999). Another protein kinase family in plants is the SNF1 family (Mikolajczyk 2000), which contains the SnRK2 subfamily. Kobayashi et al. (2004) implicated this subfamily in hyperosmotic stress signaling in rice, and a salicylic acid-, drought-, and salt-induced protein kinase from the same family was found to be an osmotic stress-activated kinase in tobacco (Mikolajczyk et al. 2000).

Phosphatases also play an important role in osmotic stress signaling cascades. Ulm et al. (2002) identified an MAPK phosphatase 1 (MKP1) from *Arabidopsis*, and a *mkp1* mutant shows increased tolerance to salinity. It is suggested that the MKP1 protein has a role in integrating and fine-tuning responses to environmental challenges by selective phosphatase activity in stress signal pathways. In *Arabidopsis*, a major class of phosphatases is the type 2C class (PP2C), which in *Arabidopsis* comprises a large protein family encoded by 76 genes (Schweighofer et al. 2004). Two of these, the ABI1 and ABI2 proteins, act in a negative regulatory feedback loop of the ABA signaling pathway and contribute nearly 50% of ABA-induced PP2C activity (Merlot et al. 2001). Mutations in both genes reduce the ABA-induced rise in Ca^{2+} in guard cells (Allen et al. 1999), highlighting a role for dephosphorylation in an ABA-mediated response to water stress. Ten PP2C transcripts have also been isolated from the halophyte *Mesembryanthemum crystallinum*, and some are induced by salinity and drought stress (Miyazaki et al. 1999).

Phosphorylation may not only play a role in signaling processes but also be important for the protective function of desiccation-related late embryogenesis abundant (LEA) proteins. This particular aspect is discussed in the section describing LEA proteins.

5.5.2 Phospholipid Signaling

In plants, phospholipid signaling is thought to play a role in many developmental processes and environmental responses, particularly via the activity of phospholipase C (PLC) and phospholipase D (PLD) (Munnik et al. 1998, Bargmann and Munnik 2006). Both PLC and PLD enzymes are lipid-degrading enzymes; PLC cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into soluble inositol-3-phosphate (IP₃) and membrane-bound 1,2-*sn*-diacylglycerol (DAG), while PLD cleaves membrane phospholipids into a polar head group and phosphatidic acid (PtdOH). PtdOH may then activate PLC (English et al. 1996). Both PLC and PLD are important in lipid catabolism and initiate

a lipolytic cascade involved in membrane disintegration during stress injury (Chapman 1998). A drought- and salt-inducible PLC gene was isolated from *Arabidopsis* (Hirayama et al. 1995) and shown to have functional phosphatidylinositol hydrolyzing activity. The involvement of PLC during dehydration has been shown in other plants such as potato (Kopka et al. 1998), pea (Venkataraman et al. 2003), and *Brassica napus* (Novotna et al. 2000). The product of PLC degradation, IP3 is a second messenger that releases Ca^{2+} from internal stores necessary for the control of stomatal aperture by ABA in tobacco (Hunt et al. 2003). There is further evidence that the second messenger IP3 is involved in plant stress responses to ABA. This arises from analysis of the *fiery* mutant of *Arabidopsis*, which is altered in its sensitivity to salt and drought stress and to ABA (Hunt and Gray 2001). The *FIERY* gene encodes inositol phosphate 1-phosphatase, which catabolizes IP3. *fiery* mutant plants therefore have approximately 10 times the wild-type IP3 level as do wild-type plants; this leads to high-level expression of ABA/stress-induced genes, identifies *FIERY* as a negative regulator of ABA and stress signaling in *Arabidopsis* (Xiong et al. 2001), and underlines the importance of IP3 for ABA/stress responses. Antisense expression of *AtPLC1* in *Arabidopsis* leads to reduced IP3 levels following ABA stimulation and the opposite scenario in *fiery* mutants, namely a decreased sensitivity to ABA (Sanchez and Chua 2001). This further confirms a role for IP3 action in ABA stress signaling.

PLD genes have been isolated from several species and transcript expression and enzymatic activity correlated with drought stress for cowpea (El Maarouf et al. 1999) and for *C. plantagineum* (Frank et al. 2000). In *Arabidopsis*, PLD is encoded by a multigene family (Wang et al. 2000) of which the *PLD Δ* transcript accumulates significantly in response to dehydration and salt stress (Katagiri et al. 2001). That the accumulation of PtdOH is linked to PLD activity in response to dehydration was shown by antisense PLD transgenic plants that showed reduced PtdOH accumulation (Katagiri et al. 2001). Similarly, transgenic *Arabidopsis* plants for *PLD Δ* also showed decreased levels of PtdOH and an increase in superoxide production (Sang et al. 2001a). Additionally, *PLD α* -depleted plants showed enhanced transpiration and decreased drought stress tolerance. In the same study, *PLD α* overexpression led to an enhanced sensitivity to ABA (Sang et al. 2001b), supporting another study showing PLD has a role in mediating ABA-promoted guard cell closure in response to water stress (Jacob et al. 1999).

5.5.3 Other Signaling Molecules

Several additional molecules have been shown to have a possible signaling role in plant responses to environmental stresses. One such molecule is the

polyamine spermidine; when the spermidine content of *Arabidopsis* leaves was raised following constitutive overexpression of the spermidine synthase gene from *Cucurbita ficifolia*, plants showed an enhanced tolerance to drought and salt stress, including upregulation of stress-responsive proteins such as DREB and rd29A (Kasukabe et al. 2004), suggesting spermidine may act as a signaling regulator in stress signaling pathways.

Nitric oxide accumulation has also been observed in several plant species (Barosso et al. 1999), and some studies have placed it as a signaling molecule within adaptive responses to drought stress. Mata and Lamattina (2001) showed that exogenous application of nitric oxide donors in *Tradescantia*, *Salpichroa*, and *Vicia* caused increased stomatal closure, decreased ion leakage, and increased LEA protein transcripts after drought exposure. Another study has shown that nitric oxide selectively regulates Ca^{2+} -sensitive ion channels in *Vicia* guard cells by raising cytosolic free Ca^{2+} concentration (Garcia-Mata et al. 2003), thereby placing it within a branch of ABA-mediated Ca^{2+} signaling pathways in guard cells, relevant for drought stress.

5.5.3.1 Absciscic Acid Mediates Gene Expression. ABA is recognised as the major plant hormone involved in integrating environmental changes in water availability with adaptive responses in plants (Koornneef et al. 1998). Drought and salt stress responses are mediated by a massive ABA-mediated alteration of gene expression (Hoth et al. 2002, Seki et al. 2002). Not only do ABA levels rapidly increase following water stress, but exogenous ABA treatment can mimic the induction of genes observed following stress treatment, as exemplified by treated callus from the resurrection plant *C. plantagineum* (Bartels and Salamini 2001). Among a range of other physiological processes in plants, ABA also plays a role in the acquisition of desiccation tolerance in seeds (reviewed in Leung and Giraudat 1998) and, relevant to osmotic stress, the regulation of stomatal closure (reviewed in Schroeder et al. 2001). The control of ABA biosynthesis in plants is complex (reviewed in Taylor et al. 2000, Seo and Koshiba 2002): the identification and cloning of genes involved in ABA biosynthesis and signaling have been instructive in understanding ABA signal transduction. A key ABA biosynthetic enzyme is 9-*cis*-epoxycarotenoid dioxygenase (NCED), which has been shown to be drought inducible in several plant species, including *Arabidopsis*, cowpea, and tomato (Iuchi et al. 2000, 2001, Thompson et al. 2000). Transgenic studies overexpressing the endogenous NCED gene in *Arabidopsis* produced plants with improved drought tolerance and enhanced ABA levels (Iuchi et al. 2001), demonstrating that it is possible to modulate drought tolerance by the engineering of critical steps in the ABA biosynthetic pathway.

Mutants in ABA biosynthesis have been isolated in several plants species (Seo and Koshiba 2002) and other classes of mutants in *Arabidopsis*, including

ABA-hypersensitive and -insensitive mutants such as *abi1* and *abi2*. The *ABI1* and *ABI2* loci encode protein phosphatases (Meyer et al. 1994, Leung et al. 1997), indicating that protein phosphorylation has a role in ABA signaling pathways and, by implication, in stress-responsive pathways. It is assumed, although not proved, that the site of ABA perception is the plasma membrane (Zhang et al. 2002) and that a soluble ABA receptor exists (Zhang et al. 2001). There is a large array of ABA signal receptors, including G proteins, protein phosphatases, and protein kinases of the calcium-dependent protein kinase (CDPK) class and sucrose nonfermenting (SNF-1) group (Federoff 2002). The control of ABA signaling has been well reviewed (see Himmelbach et al. 2003) and is not repeated here.

A role for other plant hormones has been implicated in ABA action: it is known that ABA can finetune growth via ethylene action (Chen et al. 2002, Sharp and LeNoble 2002). Furthermore, another stress- and ABA-inducible SNF1-like protein kinase *PKABAI* from wheat was shown to mediate ABA-promoted suppression of gene induction by gibberellins (Gómez-Cadenas et al. 1999).

5.6 Molecular Responses: Gene Expression in Response to Dehydration and Desiccation

Processes that control plant dehydration tolerance are regulated at the molecular level. It is hypothesized that abiotic stress tolerance will be due to differential accumulation of genes common to all plants, gene products with modified activity, or the presence of unique genes (Ingram and Bartels 1996). With the advent of large-scale insertional mutagenesis technology, the development of high-throughput molecular techniques, and the completion of genome sequencing programs, molecular genetic analysis has entered a new era. Many genes have been reported as playing a role in dehydration tolerance, and the functions of some gene products has been established (Zhu 2002).

Plants respond to environmental cues such as drought by modifying gene expression patterns, which may or may not lead to stress tolerance. Early work using differential screening techniques revealed that steady-state mRNA levels are altered in response to drought treatment (see reviews in Bray 1993, Zhu et al. 1997). The advent of transcript cataloguing and the use of DNA microarrays have provided a greater insight into the dynamic changes in gene expression that occur as a consequence of environmental stimuli. Transcriptome analysis has concentrated mainly on genetic model species (i.e., *Arabidopsis* and rice); however, despite access to the complete genome sequence, the full

set of expressed RNA molecules has yet to be determined. Open-ended format technologies such as serial analysis of gene expression (SAGE; Robinson et al. 2004) and massively parallel signature sequencing (MPSS; Meyers et al. 2004) will enable a global (i.e., full transcriptome) analysis of drought-responsive gene expression in the future. At present, relatively large collections of genes have been surveyed, and it is apparent that many changes in gene expression take place.

In cases where changes in steady-state mRNA levels occur, broadly, three basic patterns of gene expression are observed in response to drought: class 1 transcripts, which accumulate during the stress period but disappear during recovery; class 2 transcripts, which accumulate during the very early stages of stress but only transiently; and class 3 transcripts, which are downregulated during stress. One hypothesis is that the gene expression profile correlates with the function of the gene product. In that, class 1 genes play a role in protecting/stabilizing plant cell structure, class 2 genes have a regulatory function, and class 3 genes are involved in physiological responses that are downregulated by stress such as photosynthesis. To some extent, this hypothesis is not refuted; however, classification on the basis of this premise is not straightforward because genes are often developmentally regulated and respond to other stimuli.

Specific gene expression profiles have been reported in response to drought conditions, but there are conflicting reports concerning the similarity between the observed transcriptome changes. Seki et al. (2002) reported a very strong correlation between genes induced by high salinity and those induced by drought stress using an *Arabidopsis* full-length cDNA microarray. In contrast, the study by Kreps et al. (2002) indicated that the majority of transcriptome changes were stimulus specific and not part of a general stress response common to osmotic and salt stress. The reason for the discrepancy may be due to differences in the sensitivity of the array technologies used in the *Arabidopsis* studies (Kreps et al. 2002).

Of the class 1 genes that have been reported, the LEA protein genes are of particular interest because there is increasing evidence that they act as osmoprotectants under stress conditions (Cuming 1999, Wise and Tunnacliffe 2004). *LEA* genes are induced in many species, including *Arabidopsis* and rice, in response to dehydration and to other osmotic stimuli. In *C. plantagineum*, the effects of salt and dehydration on transcript accumulation patterns were studied by Smith-Espinoza et al. (2003). Expression analysis revealed dehydration-specific profiles of *LEA* genes. Given that *C. plantagineum* is desiccation tolerant, but sensitive to relatively low levels of sodium chloride, this again suggests that dehydration-specific responses involve synchronized gene expression and that the regulation of gene expression is important to acquire tolerance.

5.6.1 Late Embryogenesis Abundant Genes and Proteins

LEA genes and proteins are associated with the acquisition of desiccation tolerance in seeds and resurrection plants. In all higher plants, LEA proteins accumulate in the late phase of embryogenesis concomitant with the onset of seed desiccation tolerance (Dure et al. 1989). LEA proteins are also upregulated in vegetative tissues of a wide range of desiccation-sensitive plants following dehydration stress (Ingram and Bartels 1996). LEA proteins can be divided into different groups based on conserved sequence motifs (Wise 2003). Experimental evidence that LEA proteins directly protect cellular structures or ameliorate the consequences of drought stress is scant, although it includes evidence from transgenic plants and from in vitro protection assays (Bartels and Sunkar 2005). The facts that LEA proteins are widely distributed among monocot and dicot species, share highly conserved domains, and are an important component of the acquisition of seed desiccation tolerance and that their developmental specificity following drought stress strongly imply a fundamental role in drought stress (Wise and Tunnacliffe 2004). This is strengthened by the recent finding that an LEA protein is upregulated in response to desiccation in the anhydrobiotic nematode *Aphelenchus avenae* (Browne et al. 2002) and suggests that LEA proteins play a protective role against anhydrobiosis in widely divergent organisms. Because the majority of LEA proteins are cytosolic, it has been proposed that the coiled domains of certain LEA proteins may have a role in binding water to perhaps maintain a minimum cellular water requirement or that others may be involved in the “solvation” of cytosolic structures by providing a stable cohesive protective layer (Baker et al. 1988). One class of LEA proteins, the dehydrins, have been shown to have detergent and chaperone-like properties that stabilize membranes and protein cellular components (Close 1996). Some LEA proteins are known to associate with cell membranes and have been suggested to prevent liposome leakage on desiccation (Sales et al. 2000).

Studies on LEA protein expression demonstrate the involvement of LEA proteins in dehydration responses of drought-sensitive plants but also the involvement of LEA proteins in desiccation tolerance. A comparison of the LEA proteins expressed in sensitive and tolerant plants does not point to qualitative differences of the LEA proteins. In contrast, the LEA proteins in all plants are evolutionary conserved and contain common, diagnostic motifs. Analysis of LEA proteins in the resurrection plant *C. plantagineum* suggests that differences may be quantitative rather than qualitative when LEA gene expression is compared between *C. plantagineum* and a sensitive plant like *Arabidopsis thaliana* (Bartels et al. unpublished data). As described in detail in Bartels et al. (2006), many different classes of LEA and LEA-like proteins are expressed with a comparable expression profile in *C. plantagineum*. A survey of the *Arabidopsis* genome revealed 53 LEA and LEA-like genes, which

can be subdivided in nine different groups, with group 3 being the most abundant group, followed by the dehydrin group 2 and the six members of the seed maturation proteins of group 6. An expression analysis of the transcripts encoded by the *LEA* genes showed an abundant expression in mature seeds for 50% of the genes and 30% increased in abundance in vegetative tissues in response to osmotic stress (D. Hinch and M. Hundertmark, personal communication). Interestingly, only a minimal overlap was found for genes expressed in seeds and osmotically stressed vegetative tissues.

Studies of *LEA* gene expression in *C. plantagineum* and tolerant as well as sensitive relatives suggest that some differences can be found in promoter regions (D. Bartels, unpublished data). The differences, which are observed in the promoter structures, may lead to higher expression levels of a dehydrin-type *LEA* gene in desiccation-tolerant plants than in desiccation-sensitive plants (D. Bartels, unpublished data). This hypothesis is currently being tested experimentally.

5.6.1.1 Phosphorylation of *LEA* Proteins. Several publications report the phosphorylation of *LEA* proteins in seeds as well as in vegetative tissues of resurrection plants. Examples of *LEA* proteins, which are known to be phosphorylated, include the ABA-responsive maize embryo RAB17 protein, whose *in vivo* phosphorylation (Vilardell et al. 1990) affects its binding properties to peptides with nuclear localization signals (Goday et al. 1994). In addition, the early response to dehydration (ERD)14 protein is a dehydrin with ion binding properties, which are dependent on phosphorylation (Alsheikh et al. 2003 2005). Röhrig et al. (2006) reported that two abundantly expressed groups of *LEA* proteins undergo reversible phosphorylation during dehydration in leaves and roots of the resurrection plant *C. plantagineum*. These are the dehydrin-type *LEA* proteins and the *LEA*-like proteins CDeT11-24. The phosphorylation sites in CDeT11-24 proteins coincide with predicted coiled-coil regions, which leads to the hypothesis that the phosphorylation could alter the stability or specificity of interactions of this group of *LEA* proteins with other molecules possibly proteins and also carbohydrates; the interactions may be important in stabilizing cellular structures during desiccation.

5.6.2 Expression of Dehydration-Induced Genes Other Than *LEA* Genes

A general database survey of transcripts induced by dehydration in desiccation-sensitive and -tolerant plants does not, with a few exceptions, identify genes that are specifically induced in desiccation-tolerant plants. This statement has to be considered with caution, as no full genome analysis from

a desiccation-tolerant plant is available. Collett et al. (2004) compared cDNAs expressed in the desiccation-tolerant plant *Xerophyta humilis* with desiccation-sensitive plants and found mainly genes commonly associated with dehydration responses. A few transcripts were identified that were previously not associated with water deficit, but the significance of those transcripts is difficult to evaluate. These published data are supported by comparative transcript analysis in *C. plantagineum* with transcripts expressed in desiccation-sensitive and desiccation-tolerant close relatives of *C. plantagineum* (Bartels et al. unpublished data). Further comparisons of *C. plantagineum* and closely related plants indicate that control elements in promoter regions may contribute to differences in tolerant and sensitive plants (D. Bartels, unpublished data). A similar observation has been reported by Neale et al. (2000), who also elude to unique gene regulation processes, which may allow selective expression of drought-responsive genes.

5.6.3 *Transcriptional Regulation of Genes Induced by Water Deficit*

Transcriptional regulation is mediated by transcription factors via interaction with specific *cis*-acting elements located in gene promoter regions. Analysis of gene regulation in desiccation-tolerant plants has so far mainly revealed only those factors that are known from studying gene expression in response to dehydration. Therefore, the main components will be summarized. Dehydration-responsive genes are regulated by both ABA-dependent and ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000). *cis*-elements and transcription factors involved in dehydration- and salt-induced gene expression have been studied extensively: dehydration responsive element (DRE) with DRE binding (DREB) factors (Yamaguchi-Shinozaki and Shinozaki 1994, Liu et al. 1998), ABA-responsive element (ABRE) with bZIP factors termed ABRE binding factors (ABF/AREB) (Marcotte et al. 1989, Uno et al. 2000), and MYC and MYB factors with their individual recognition elements in the promoter of a dehydration and ABA-induced gene *rd22* (Abe et al. 1997, Goh et al. 2003). Other transcription factor families, such as NAC, WRKY, RING finger, and zinc finger genes, have been associated with abiotic stress responses (Chen et al. 2002, Seki et al. 2002, Fujita et al. 2004).

In early models, ABA-independent stress-responsive gene expression was thought to be regulated through DRE *cis*-elements, while ABA-dependent pathways activate gene expression through ABRE *cis*-elements (Shinozaki and Yamaguchi-Shinozaki 1997). In ABA-independent pathways, *Arabidopsis* AP2-type transcription factors (DREB2A and DREB2B) *trans*-activate the DRE *cis*-element of osmotic stress-responsive genes (Liu et al. 1998).

However, analysis of a *DREB*-type transcription factor, *CBF4* in *Arabidopsis*, showed that regulation of DRE elements may also be mediated by an ABA-dependent pathway. *CBF4* gene expression is upregulated by drought and ABA, but not by cold stress. Overexpression of *CBF4* in *Arabidopsis* resulted in constitutive expression of DRE-containing stress-responsive genes and enhanced tolerance to drought and freezing stresses (Haake et al. 2002).

The cross-talk between ABA-dependent and ABA-independent pathways is further illustrated by the activity of homeodomain leucine zipper (HD-Zip) class transcription factors. HD-Zip factors are of particular interest because they have been found exclusively in the plant kingdom, which means they are likely to be involved in processes that are unique to plants, such as responses to environmental perturbations (Ruberti et al. 1991, Söderman et al. 1994). Several members were linked to environmental adaptation by studying gene expression patterns or analysis of transgenic and mutant plants. *ATHB6* (Söderman et al. 1999, Himmelbach et al. 2002), *ATHB7* (Söderman et al. 1996, Olsson et al. 2004), and *ATHB12* (Lee et al. 2001) from *Arabidopsis* and *CpHB-1*, -2, -6, and -7 (Deng et al. 2002, Frank et al. 1998) from *C. plantagineum* were linked to drought responses. The activity of HD-Zips resides primarily in the specific DNA binding property of the HD and the ability of the leucine zipper to mediate protein–protein interaction with other HD-Zips, thus expanding the number of potential target genes that may be transcriptionally regulated. *CpHB-1* is capable of homodimerization and dimerization with a second dehydration responsive HD-Zip, *CpHB-2*. In contrast to *CpHB-1*, *CpHB-2* is responsive to exogenously applied ABA, leading to the suggestion that they act in different branches of the dehydration-induced signaling network (Frank et al. 1998).

A striking similarity has been observed in the transcriptional regulation of *CpHB-1* and *ATHB5*, an *Arabidopsis* HD-Zip that regulates ABA responsiveness. Both the *CpHB-1* and *ATHB5* promoters direct gene expression in a tissue-specific manner: transcriptional activity was found in the transition zone, which connects cotyledons and the top of the radicle/root in young seedlings and associated with vascular tissue (Johannesson et al. 2003, Deng et al. 2006). The gene products vary in the structure of leucine zipper protein–protein interaction domain, which appears to affect the functional properties of each protein. Ectopic expression of *CpHB-1* lead to ABA insensitivity (Deng et al. 2006), whereas overexpression of *ATHB5* caused enhanced sensitivity to the inhibitory effect of ABA on seed germination and seedling growth. Thus, both *ATHB5* and *CpHB-1* act as regulators of ABA responsiveness, with each using a different molecular mechanism.

A study of the physiological function of the *Arabidopsis* protein kinase CIPK1 and its role in signaling networks in abiotic stress responses revealed a convergence point for ABA-dependent and ABA-independent stress

responses on the protein level (D'Angelo et al. 2006). The analysis provides evidence for alternative protein complex formation as a mechanism integrating signals and generating output specificity in plant abiotic stress responses. It was shown that the kinase CIPK1 forms complexes either with CBL1 or CBL9 and, depending on the interaction partner, CIPK1 osmotic stress sensitivity affects ABA responsiveness. This possibility for alternative complex formation makes CIPK1 as a control point for two different stress pathways (D'Angelo 2006).

Abiotic stress may also bring about changes in chromatin structure, which may in turn alter the accessibility of transcription factors to gene promoter regions. This is supported by the discovery of *CpR18* from *C. plantagineum*. *CpR18* encodes a protein with specific binding activity for a *cis*-acting element in the promoter of the ABA and dehydration responsive gene *CDeT27 45* (Hilbricht et al. 2002). The protein contains an acidic region, an SAP domain, a zinc finger of the C3H type, and two motifs that are conserved in proteins from several plant species. One of the conserved regions is rich in basic residues and is predicted to form a helix-loop-helix structure. The SAP domain is a eukaryotic module present in proteins associated with transcription, chromatin modeling, and nucleic acid repair (Aravind and Koonin 2000, Sachdev et al. 2001). For SAP domains from humans, *Caenorhabditis elegans*, *Schizosaccharomyces pombe*, and *Arabidopsis*, sequence-specific interactions with scaffold attachment region DNA have been demonstrated (Kipp et al. 2000). In plants, detailed analysis of *Arabidopsis* poly(ADP-ribose) polymerase showed that the SAP domain is essential and sufficient for association with chromatin *in vivo* and mediates formation of stable dimers that bind nucleic acids (Babychuk et al. 2001).

Downregulation of genes has not been considered here as an essential component of preparing plants for desiccation. All investigations have shown that photosynthesis-related and energy-related gene expression programs are downregulated when desiccation tolerance is expressed (Bartels and Salamini 2001, Collett et al. 2004).

5.6.4 Translational Control During Osmotic Stress

Differential selection of mRNA for translation was shown to be a widespread component of gene regulation in response to environmental stress (Kawaguchi et al. 2004). Steady-state mRNA levels do not necessarily correlate with the corresponding activity or abundance of proteins in biological tissues. Dehydration results in a reduction in protein synthetic capacity, which is manifested by a loss in polysomes (Kawaguchi *et al.* 2004). In this study, 28% of the genes surveyed showed no significant change in ribosomal loading, and a small proportion (<1%) of the mRNAs showed a significant increase in

ribosomal loading in response to dehydration. The remaining nontranslated fractions form messenger ribonucleoprotein particles (mRNPs). Some mRNAs showed little or no change in abundance but a significant reduction in polysome association, such as ribosomal protein mRNA. The shift of ribosomal protein mRNAs to the nontranslated RNA fraction in response to dehydration indicates that they are stable when not translated. The sequestration of transcripts into mRNPs to alter mRNA stability is a mechanism used by the desiccation-tolerant *Tortula ruralis* in response to rapid desiccation (Wood and Oliver 1999). This strategy may allow for rapid recovery of normal physiological processes after stress is alleviated (Phillips et al. 2002, Kawaguchi et al. 2004).

The mechanisms that link RNA metabolism and translation in plants under osmotic stress are unclear. The identification of mutants with altered ABA responses that involve genes encoding nuclear RNA-binding proteins and noncoding RNAs [see “Craterostigma Desiccation Tolerant (*CDT*) Genes”)] will help unravel how post-transcriptional events affect mRNA translation during osmotic stress.

5.6.4.1 Transketolase Genes in *C. plantagineum*. This phenomenon of translational control has been reported for two transketolase transcripts (*tkt7* and *tkt10*) that were isolated from *C. plantagineum* and preferentially associated with rehydrating tissues (Bernacchia et al. 1995). Despite an abundant level of *tkt7* and *tkt10* transcripts in rehydrating leaves, proteins could not be isolated. This was shown to be due to translational control mechanisms acting on the loading of mRNAs to polysomes. Transketolase gene expression in *C. plantagineum* may be a particular case of gene duplication during the process of acquisition of desiccation tolerance. So far three differentially expressed transketolase genes have not been reported from any higher plant except for *C. plantagineum*, and the generation of more transketolase genes in new pathways may be an example for an adaptation mechanism. In most higher plants, the only known form of the transketolase gene is the gene encoding the transketolase of the Calvin cycle. The gene is nuclear encoded and the protein is transported to plastids. Research in *C. plantagineum* revealed that one major form of transketolase exists that corresponds to the form occurring in other higher plants, but besides that, two other forms, *tkt7* and *tkt10*, exist that are most probably not localized in the chloroplast (Bartels et al., unpublished data, Bernacchia et al. 1995). It appears that the existence of these two other forms is linked with the unusual carbohydrate metabolism in *C. plantagineum*. Fully hydrated leaves of *C. plantagineum* contain high levels of octulose, which is converted to sucrose during dehydration, and the process is reverted during rehydration (Bianchi et al. 1991). The octulose sucrose cycle requires the expression of particular transketolase genes that may have been established by gene duplication when desiccation tolerance was acquired in

C. plantagineum. So far this has not been reported for any other desiccation-tolerant plants. It will be interesting to know whether similar transketolase isoforms exist in other desiccation-tolerant plants that have an octulose sucrose cycle. It is suggested that the octulose sucrose conversion is an important component of desiccation tolerance in *C. plantagineum*.

5.7 *Craterostigma* Desiccation Tolerant (CDT) Genes

Insertional mutagenesis experiments have been successful in discovering components of dehydration tolerance signaling pathways. Loss-of-function and gain-of-function mutants with differing sensitivity to stress have been isolated, and the disrupted loci have been identified and characterized (Zhu 2002). In some cases, the gene products share homology with genes that have been well characterized in other systems, whereas others appear to be novel. To illustrate the power of this genetic approach, an example from the desiccation-tolerant plant *C. plantagineum* is described here: a novel gene family from *C. plantagineum* that appears to encode non-protein-coding RNAs and functions as ABA signaling molecules.

A genetic approach has been used to identify intermediates in the ABA signal transduction pathway leading to desiccation tolerance in *C. plantagineum*. tDNA activation tagging experiments permitted the isolation of a gene (*CDT-1*), which encodes a signaling molecule in the ABA transduction pathway (Furini et al. 1997). Constitutive expression of *CDT-1* led to desiccation tolerance in the absence of ABA and to the constitutive expression of a subset of dehydration and ABA responsive transcripts. *CDT-1* is a novel gene that has features of a short interspersed element (SINE) retrotransposon. The mechanism by which *CDT-1* expression results in desiccation tolerance is unclear because the mRNA does not code for a polypeptide greater than 22 amino acids in length.

Using a similar tDNA tagging technique, a second mutant (*cdt-2*) was identified that possesses significant sequence similarity to *CDT-1* (Smith-Espinoza et al., unpublished data). In this study, transcriptional activation of a truncated version of the *CDT-2* gene that lacked the peptide-coding region identified by Furini et al. (1997) was sufficient to confer desiccation tolerance. Consequently, it is proposed that *CDT-1/2* genes function as regulatory noncoding (nc) RNA molecules. ncRNA genes produce functional RNA molecules rather than encoding proteins. However, almost all means of gene identification assume that genes encode proteins, so even in the era of complete genome sequences, ncRNA genes have been effectively invisible (Eddy 2001). ncRNAs are thought to play roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of

gene expression or in guiding RNA modifications. The connection between ABA signaling and RNA metabolism was also highlighted by the discovery of dysfunctional alleles of *abscisic acid hypersensitive 1* (*ABH1*; Hugouvieux et al. 2001), *hyponastic leaves 1* (*HYL1*; Lu and Fedoroff 2000), and *super-sensitive to ABA and drought 1* (*SAD1*; Xiong et al. 2001) genes, which encode RNA binding proteins.

5.8 Integration of Genetic Pathways: MAP Kinase Cascades

Pathway integration during abiotic stress signaling in plants can potentially occur at many levels. Recent studies have demonstrated that MAPK cascades may play a pivotal role in the integration and finetuning of plant responses to various environmental challenges, including salt and drought stress. This is primarily due to the complex network of possible interactions and the fact that MAPK genes exist as large gene families (Jonak et al. 2002). Signals perceived by the MAPKK kinases (MAPKKKs) have to be transduced through MAPKKs, to MAPKs, which offer scope for cross-talk between different stress signals. MAPKs are implicated in abiotic stress signaling (Ligterink and Hirt 2001). A putative MAPK cascade involved in osmotic stress signaling was discovered in *Arabidopsis* and consisted of AtMEKK1 (MAPKKK), AtMEK1/AtMKK2 (MAPKK), and AtMPK4 (MAPK) (Ichimura et al. 1998, 2000). More recent data demonstrated that AtMKK2 was specifically activated by both salt and cold stress and by AtMEKK1 (Teige et al. 2004). Plants over-expressing *AtMKK2* exhibited constitutive AtMPK4 and AtMPK6 activity, constitutively upregulated expression of stress-induced marker genes, and increased freezing and salt tolerance. Transcriptome analysis of *AtMKK2*-overexpressing plants demonstrated altered expression of 152 genes involved in transcriptional regulation, signal transduction, cellular defense, and stress metabolism. This provides strong evidence for the involvement of MAPKKK-MAPKK-MAPK modules in the integration of abiotic stress signaling cascades.

5.9 Oxidative Damage and Reactive Oxygen Species

Sufficient defense mechanisms against oxidative damage appear to be essential in acquisition of desiccation tolerance. Drought stress causes the accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals (Hasegawa et al. 2000, Apel and Hirt 2004). The main site of ROS production and first target of ROS damage is the chloroplast (Allen 1995). However, photorespiratory activity is also increased by drought and causes elevated levels

of glycolate-oxidase activity resulting in H_2O_2 production (Mittler and Zilinskas 1994). ROS contribute to cellular damage, because transgenic plants overexpressing ROS scavengers show enhanced tolerance to multiple abiotic stresses (Roxas et al. 1997, Allen 1995, Nuccio et al. 1999 [review]). ROS may be seen as cellular indicators of stress, and overaccumulation of ROS can result in cell death (Asada 1999). However, they may also act as signals, not only to induce ROS scavengers but also to potentially activate downstream cascades via Ca^{2+} (Price et al. 1994), and may be directly sensed by signaling proteins such as a tyrosine phosphatase (Xiong and Zhu 2002). This dual effect of ROS in being toxic but also participating in signaling suggests that plant cells require different mechanisms to, on the one hand, detoxify excesses of ROS during stress and, on the other, to maintain low levels of ROS during stress for signaling.

In the ascorbate-glutathione cycle, two key enzymes are thought to detoxify reactive oxygen species in plants. The primary scavenger SOD converts superoxide to hydrogen peroxide. Hydrogen peroxide together with metal reductants form highly reactive and therefore toxic hydroxyl radicals via the Haber-Weiss or Fenton reaction (Asada and Takahashi 1987). Therefore, its detoxification by ascorbate peroxidase (APX) and catalase (CAT) is of key importance. Both SOD and CAT enzymes exist as multiple isozymes in the chloroplast and cytosol (Asada 1994). It has been shown in many plants that dehydration alters the amounts, activities, and steady-state mRNA levels of SOD and APX. The correlation between a functional antioxidant system and ability to survive desiccation is shown by the resurrection plant *Myrothamnus flabellifolia*, which can survive 4 months of desiccation and rehydrate, but not 8 months, after which time the antioxidant system is broken down (Kranter et al. 2002). Although the major enzymatic antioxidants in plants are SOD, APX, and CAT, nonenzymatic antioxidants include ascorbate, glutathione, flavanoids, and carotenoids (see Apel and Hirt 2004).

Recent studies have highlighted yet further novel detoxifying components relevant to dehydration stress. Aldehydes are highly reactive molecules produced as a result of free radical-mediated lipid peroxidation in plants. An ABA- and dehydration-inducible aldehyde dehydrogenase (ALDH) from *C. plantagineum* and its *Arabidopsis* homologue have been described (Kirch et al. 2001). Transgenic *Arabidopsis* plants overexpressing the *Arabidopsis* *ALDH3* gene showed improved tolerance to a range of abiotic stresses including dehydration and salt (Kotchoni et al. 2006, Sunkar et al. 2003), confirming its role in a novel functional detoxification mechanism. In addition, another class of enzymes, aldose/aldehyde reductases, which reduce aldehydes and ketones, have also been shown in transgenic studies to be effective in protecting plants against oxidative damage due to heavy metals and paraquat (Oberschall et al. 2000). An aldose reductase gene from the resurrection plant *Xerophyta viscosa* was also cloned and implicated in increased osmotic stress

tolerance in bacteria (Mundree et al. 2000). Yet another novel dehydration-induced antioxidant enzyme, a 1-Cys peroxiredoxin, was isolated from *Xerophyta viscosa* (Mowla et al. 2002), thought to protect DNA against peroxide damage. The resurrection plant *Myrothamnus flabellifolius* contains a high concentration of the polyphenol 3,4,5-tri-O-galloylquinic acid in leaves, which increases to 74% by weight in dehydrated leaves and was shown to protect membranes from free radical-induced oxidation during desiccation (Moore et al. 2005).

It has been shown that membranes from desiccation-tolerant seeds are less sensitive to ROS than are those from sensitive seeds, showing that the regulation of membrane composition and especially antioxidant components plays a role in desiccation protection (Senaratna et al. 1985).

5.10 Conservation of Pathways

It appears that molecular responses are common between species from widely different phylogenetic groups and that gene regulatory cascades are conserved. Much of the evidence to support this theory is derived from large-scale expression analysis of *Arabidopsis* and rice genes during periods of abiotic stress (Rabbani et al. 2003). Rabbani et al. (2003) reported a common set of 35 salinity and drought inducible *Arabidopsis* and rice genes, of which 24 were also ABA inducible. Analysis of the putative promoter regions revealed the presence of consensus DRE- and ABRE-type *cis*-elements, supporting the hypothesis that functionally equivalent DREB and ABF/AREB transcription factors are active in regulating abiotic stress tolerance in *Arabidopsis* and rice (Hobo et al. 1999, Uno et al. 2000, Dubouzet et al. 2003). Based on these observations, it is likely that subtle differences in gene expression patterns brought about through connections between *cis*-acting elements and transcriptional regulators accounts, at least in part, for the wide variation in salinity and drought tolerance observed in the plant kingdom.

5.11 Conclusions

It is apparent from the analysis of different processes that desiccation tolerance affects gene expression in many different pathways. A combination of two possible mechanisms may have led to acquisition of desiccation tolerance: the selection of genes with unique functions related to desiccation tolerance and the development of regulatory networks activating abundant expression of protective gene products in different pathways. An example for a unique gene is the case of the *CDT-1* gene (Furini et al. 1997). With the

exception of discussing the noncoding *CDT* genes isolated in *C. plantagineum*, this chapter has not yet considered mechanisms of selected gene expression obtained via gene silencing mechanisms involving short RNAs, although a contribution on this level is expected as recent work in *Arabidopsis* has indicated (Sunkar and Zhu 2004).

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Section 2
**Desiccation Tolerance of Pollen,
Spores, and Seeds**

6 Seed Desiccation-Tolerance Mechanisms

Patricia Berjak, Jill M. Farrant, and Norman W. Pammenter

6.1 Introduction

Plants have several strategies to deal with water deficits; desiccation tolerance is but one of these. It is a rare phenomenon in angiosperm vegetative tissues but it is common, although not ubiquitous, in seeds. Desiccation tolerance is found among mosses and pteridophytes, and it occurs in certain animals such as nematodes, bdelloid rotifers, and the encysted embryos of brine shrimps, as well as in lichens, terrestrial microalgae, and microorganisms. It appears that there are certain commonalities in the mechanisms of desiccation tolerance across the wide spectrum of life forms that demonstrate this characteristic, and so for a full understanding of the phenomenon, information from all these groups should be compared (Alpert 2005, Berjak 2006). While this chapter describes the putative major mechanisms of desiccation tolerance in seeds (the reader is referred elsewhere in this book for mechanisms of desiccation tolerance in vegetative tissues), where appropriate, reference will be made to other desiccation-tolerant systems.

Desiccation tolerance implies the ability of a plant or plant part to come into equilibrium with atmospheric relative humidity (RH) and to survive in this state for ecologically significant periods. When in equilibrium with the atmosphere, the tissue will generally have a water content in the range of 0.05 to 0.15 g H₂O • g⁻¹ dry mass (strictly speaking, this is a water concentration, the amount of water per unit dry mass) and a water potential [Ψ] ≤ -100 MPa. (Consequently, the present authors consider use of the terms *anhydrobiotic*/*anhydrobiote* or *anhydrobiosis* to be inappropriate, as these tacitly suggest the complete absence of water.) Desiccation tolerance in seeds is of particular importance, not only for its ecological consequences but because the ability of seeds of many species to survive severe dehydration has been exploited by humans to store them for relatively long periods. In fact, as organized cultivation (farming) may be considered as the basis for historic centers of civilization (with both good and bad consequences [Diamond 2005]), some form of seed storage would have become entrenched very early on. This has had, and continues to have, considerable social, economic, and conservation implications. Long-term seed storage is actually an artificial situation, in that in most natural environments a seed, once shed, may not remain in the desiccated state for very long, and if dormant, it may undergo repeated cycles of dehydration

and rehydration (Thompson 2000). Nonetheless, careful manipulation of the storage environment permits successful storage of seeds for many years (see Chapter 9).

There are similarities in desiccation-tolerance mechanisms among a range of organisms and tissue types (Alpert 2005, Berjak 2006), some important features of which are reviewed later. However, a major difference between seeds and tolerant vegetative tissue (so-called resurrection plants) is that desiccation tolerance in seeds is a programed phase of embryological development and, under natural conditions, desiccation will inevitably occur (in some cases, as a postharvest event). Desiccation in vegetative parts of plants, on the other hand, is stochastic, which may have implications in terms of sensing severe water deficits and signaling processes inducing the acquisition or development of tolerance mechanisms.

To date, many of the proposals concerning tolerance mechanisms have been based on correlative evidence and, in the case of seeds, many of these putative mechanisms are based on the presence of certain biological molecules in the desiccated state (e.g., Vertucci and Farrant 1995, Pammenter and Berjak 1999). Presently, there are suggestions concerning the mode of action of such molecules, but there is generally little direct evidence as to what they actually do. However, with the development and refinement of tools to analyse functional genomics, a greater understanding is starting to permeate the literature, although we are still some way from complete comprehension of the basis of desiccation tolerance. We are, however, beginning to know which genes are transcribed (e.g., Collett et al. 2004, Illing et al. 2005, Buitink et al. 2006, Gutierrez et al. 2006), which proteins are made (Boudet et al. 2006, Oracz et al. 2007, Ingle et al. 2007), and which metabolites are synthesized (Avelange-Macherel et al. 2006, Fait et al. 2006), and even (although to a lesser extent) the regulatory and signaling processes involved (Buitink et al. 2006, Röhrig et al. 2006). However, this information is still fragmentary, and we are far from a full understanding of the integrated whole of seed (or plant) desiccation tolerance.

6.2 Acquisition of Desiccation Tolerance

This chapter is not a review of seed development. However, for an understanding of desiccation tolerance in seeds, it is necessary to consider the ability of the seed to tolerate the drying process as well as its survival in the desiccated state, and so appropriate reference is made to developmental aspects (see, e.g., Kermode and Finch-Savage 2002). During seed development, desiccation tolerance is acquired during the mid to late stages of maturation, while reserve deposition is occurring, or shortly preceding the end of this

developmental phase. It is, however, difficult to identify the exact point of acquisition of desiccation tolerance. If developing seeds are artificially dried, the rate of drying influences the response, but this effect of drying rate differs with development stage. Immature orthodox seeds will survive slow drying but not rapid drying, whereas more mature seeds do survive rapid water loss (Kermode 1995, Kermode and Finch-Savage 2002, Lima et al. 2005). This is suggested to be because immature seeds need time to develop tolerance mechanisms, which slow drying facilitates, but rapid drying does not.

During maturation, seeds go through a series of declining water concentrations (Fig. 6.1). As water is lost, the physicochemical properties of the remaining water change (Vertucci 1990), and the biochemical processes that are possible change accordingly (Vertucci and Farrant 1995). Thus, as water concentration declines, there is a number of potentially damaging processes that could occur, and seeds must have, or acquire, mechanisms to protect against them. At relatively high water concentrations (types/hydration levels V and IV [*sensu* Vertucci and Farrant 1995, $\Psi > -3$ MPa; Fig. 6.1]), these stresses are similar to those sustained by vegetative tissue under conditions of high transpirational demand and limited water supply. This is the least studied aspect of seed desiccation but is likely to involve upregulation of “house-keeping” genes, including those coding for antioxidants, and general abiotic stress responses, that have been reported for water-stressed vegetative tissue (reviewed in, e.g., Knight and Knight 2001, Seki et al. 2001, Illing et al. 2005).

With further water loss, the seed enters a phase of “intermediate” water concentrations (type/hydration level III, $\Psi -3$ to -15 MPa). At this stage, the seed is still metabolically active in that respiration continues and protein synthesis and reserve accumulation are ongoing (Fig. 6.1). Any metabolic processes, including synthesis of protectants, must occur in this water concentration range—or at higher water concentrations—because all these processes are aqueous based. However, this is also a dangerous phase, as the potential for production of reactive/active oxygen species (ROS/AOS) remains high (Vertucci and Farrant 1995, Bailly 2004, Berjak 2006), while the lower fluidity of the aqueous phase in the tissue might reduce the efficiency of detoxifying processes. Most studies on antioxidant activity have, however, been undertaken on maturing seeds during dehydration, with only limited information being available on the nature and/or level of ROS or the activity of antioxidants in developing seeds prior to maturation drying (Bailly 2004). It is likely that there are increased levels or activities of the ubiquitous antioxidant systems (see later), but what is really required are assessments of the rate of production of ROS and their subsequent detoxification (i.e., turnover rates) rather than simply measures of antioxidant levels.

It is highly likely that seeds are not really tolerant of these intermediate water concentrations in that they probably will not survive extended periods in this condition. Walters et al. (2005) stored initially dry desiccation-tolerant

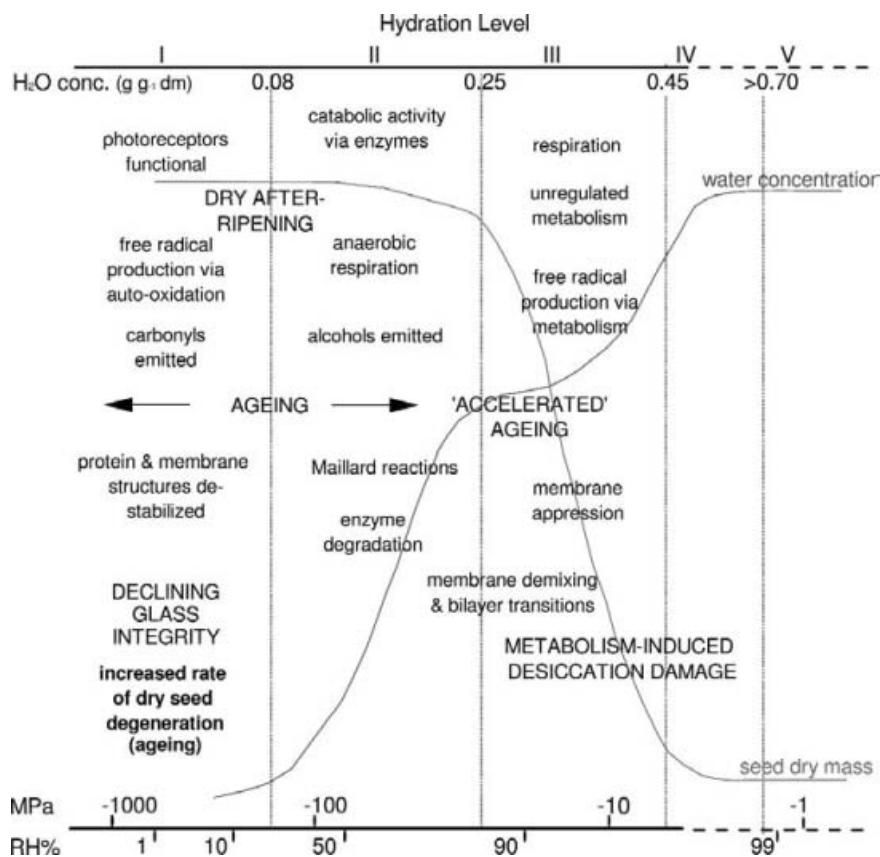


Fig. 6.1 The trends of dry mass accumulation (to a plateau) and decline in water concentration during seed maturation are depicted in relation to the events that do, or can, occur through the hydration levels III to I. Approximate water concentrations ($\text{g} \cdot \text{g}^{-1}$ dry mass) at the boundaries of the hydration levels appear at the top of the Fig., with equivalent water potentials (Ψ) and equilibrium RH values (%), below. (Modified after Vertucci and Farrant 1995, Walters et al. 2005, Berjak 2006). For color detail, please see color plate section.

seeds of several species, as well as pollen, fungal spores, some resurrection plants, and brine shrimp cysts, at relative humidities equivalent to these intermediate water contents and showed that longevity was considerably reduced. The material that those authors used had already achieved desiccation tolerance prior to the experiments. During dehydration, seeds at intermediate water contents have not yet accumulated all the protection mechanisms and thus are likely to be even more sensitive (than the material used by Walters et al. [2005]) to the time spent at these hydration levels. Under natural conditions, seeds survive passage through these intermediate water concentrations,

presumably because they are exposed to such conditions for a relatively short time.

Progression through hydration level III is characterized by several processes. For example, reserves are being accumulated, and the seed is becoming prepared for germination and, in many species, for dormancy, concomitant with development of mechanisms to tolerate desiccation (Kermode 1990, Bewley and Black 1994, Kermode and Finch-Savage 2002, Fait et al. 2006). These other processes confound the identification of the processes specific to desiccation tolerance and the determination of the role they play in achieving tolerance. Preparation for desiccation tolerance involves, *inter alia*, accumulation of sucrose and other oligosaccharides (Leprince et al. 1993, Horbowicz and Obendorf 1994, Blackman et al. 1995, Obendorf 1997, Black et al. 1999), the production of late embryogenesis abundant (LEA) proteins (e.g., Galau and Hughes 1987, Blackman et al. 1995, Russouw et al. 1995, Wolkers et al. 1998), and the appearance of novel antioxidants that are apparently unique to desiccation-tolerant organisms (Aalen 1999, Illing et al. 2005). All of these contribute to protecting the subcellular milieu (reviewed by Berjak 2006). New technology is permitting identification and semiquantitative analyses of genes, proteins, and metabolites that may well play a role in the attainment of desiccation tolerance. While these techniques show exciting promise, to date they have mostly produced lists of transcripts, proteins, and metabolites, many of which are as yet not identified and thus are presently of uncertain function (see, e.g., Baud et al. 2002, Avelage-Macherel et al. 2006, Baud and Graham 2006, Boudet et al. 2006, Buitink et al. 2006, Fait et al. 2006, Gutierrez et al. 2006, Lease and Walker 2006).

The synthesis of sugars, LEAs, and particular antioxidants having been achieved, seeds undergo maturation drying during which much of the remaining water is lost relatively rapidly, until typified by hydration level II ($\Psi < -15$ MPa; water concentration less than $0.25 \text{ g} \cdot \text{g}^{-1}$) and possibly level I, depending on the ambient RH (Fig. 6.1). The challenges facing seeds from level III and below include membrane appression, macromolecular denaturation, and continued ROS formation and accruing damage (although this might not occur as rapidly as at the higher water levels). For the seed to remain viable, these events must be prevented and, in particular, macromolecules important to germination and repair processes must be protected so that they can become operable upon rehydration.

Intracellular molecular mobility at these low water contents is considered to be limited (e.g., Walters 1998). There is, however, accumulating evidence that this water may not be uniformly distributed throughout the dry tissue (or cell), and there may be regions of higher hydration ("pockets" of water), within membranous organelles, e.g., mitochondria and plastids, in the nucleoplasm and cytomatrix, or in localized regions of the aqueous glasses (Rinne et al. 1999, Leubner-Metzger 2005, Oracz et al. 2007). This being the case, then deleterious,

protective, and metabolism-related reactions may continue to occur in these regions. For example, Elder et al. (1987) demonstrated activity of endonucleases and exonucleases in dry rye seeds; Leubner-Metzger (2005) demonstrated localized metabolic activity within inner testa cells of dry tobacco seeds; and Oracz et al. (2007) provide evidence that ROS activity in such hydrated pockets facilitates dormancy breaking in sunflower seeds. The implication of the existence of regions of higher hydration is that dehydrated desiccation-tolerant tissues are not at thermodynamic equilibrium. This state of disequilibrium could be maintained simply by the rate at which processes leading to equilibrium occur (Berjak 2006). There is also evidence of upregulation of ATPase genes during drying of desiccation-tolerant systems (Mundree and Farrant 2000, Marais et al. 2004, Buitink et al. 2006), suggesting the possibility of energy expenditure to maintain the disequilibrium. Perhaps the transition from a state of disequilibrium to one of equilibrium is coincident with death (Berjak 2006).

Before considering the nature and roles of selected categories of protectants associated with desiccation tolerance, it should be appreciated that the control processes involved in attainment of desiccation tolerance are as yet poorly understood. The reason for this is probably because signals are frequently transient and are constituted by molecular or other species that occur at such low levels as to be virtually undetectable. Furthermore, the cascade and “knock-on” effects of one process on another are rapid and difficult to detect and decipher. It is rather like looking at an underground map written in hieroglyphics!

6.3 Requirements for Desiccation Tolerance

A spectrum of requirements exists for the acquisition of desiccation tolerance and its maintenance in the dry state in seeds. These include modification of the cellular and intracellular physical characteristics to withstand physical stresses imposed by extreme degrees of dehydration; intracellular dedifferentiation; metabolic “switch-off”; the presence and efficient operation of antioxidants appropriate to the phases of dehydration, the dry state, and ultimately to rehydration; accumulation and involvement of molecules conferring protection during dehydration and in the desiccated condition; deployment (partitioning) of amphiphilic compounds; and maintenance of the integrity of repair systems required to counteract damage accumulated in the dry state, during the lag phase of imbibition that precedes germination. For reviews of the mechanisms and processes underlying these various requirements, see Vertucci and Farrant (1995), Pammenter and Berjak (1999), and Buitink et al. (2002).

The focus has been in particular on the accumulation and roles of sugars and proteins—particularly the LEAs—and on the occurrence and operation of antioxidants (reviewed in Berjak 2006), as well as on the underlying details of control, which are presented later. Before considering the integration of these factors, it is instructive to follow the historical progress that has led to their present prominence in explanations of the mechanisms conferring and maintaining desiccation tolerance.

6.3.1 *Proteins*

Two main classes of stress-associated proteins (which might actually represent two subsets of one major class of heat-stable, hydrophilic proteins) that are associated with seed development are the small heat-shock proteins (HSPs) and the LEA proteins. However, at least one other category of stress-associated protein might also exist.

There is some evidence that seed proteins traditionally accepted as serving a storage function only, might also have a role in protection against desiccation stress. For example, Mtwisha et al. (2007) have reported a 53-kDa LEA-like protein (ASP53) in *Acacia erioloba* seeds that has characteristics of both a storage protein and an LEA. This protein is heat stable, is able to prevent denaturation of enzymes, and is able to decrease the rate of temperature-dependent loss of secondary structure of haemoglobin, thus acting like a chaperonin. The ASP53 gene sequence was determined and found to contain two cupin motifs with homology to seed storage proteins such as phaseolin (Mtwisha et al. 2007). Immunocytochemical studies carried out by those authors showed the protein to be present in storage vacuoles in cotyledons, but it was located in the cell walls in the embryonic axes, thus suggesting a duality of roles. The results of Mtwisha et al. (2007) are in accord with observations of Castillo et al. (2000) that the expression of psp54, a protein of the vicilin superfamily in pea, is undetectable prior to seed desiccation (see review in Kermode and Finch-Savage 2002).

Reports on potential dual roles of storage proteins and LEAs are rarely encountered, probably because few investigations on the LEAs themselves have been conducted, their roles having been largely been inferred from RNA studies. Thus the intracellular role(s) and location(s) (Table 6.1)—mostly purported to be cytomatrical—have rarely been confirmed for the proteins themselves.

Small HSPs appear in developing seeds ahead of events associated with desiccation and are accumulated, persisting in quantity in the dry state (Vierling 1991, Coca et al. 1994, DeRocher and Vierling 1994, Wehmeyer et al. 1996). Small HSP monomers (molecular mass 15 to 42 kDa) are assembled as

Table 6.1 Classification, properties, and proposed roles of LEA proteins, reported in various sources.

Group	Superfamily (Wise 2003)	Family	Pfam Domain (Illing et al. 2005)	Motif/Consensus	Postulated Roles and Distributions (Where Applicable)
1 (I)		D-19	PF00477	GGQTRREQLG- EEGYSQMGRK	Water binding
2 (II)		D-11 dehydrins	PF00257	K segment at carboxyl terminus: EKKGIMDKI- KEKLPG or minor variants, but all characterized by KIKEKLPG S (serine-rich) segment in some dehydrins; Y segment in most—(V/T)DEYGNP— near amino terminus	All seedling tissue (water-stressed or not) b quantitative differences related to tissue type. Localized (also) near membranes of protein bodies in maize scutellum; endomembrane localization in onion epidermal cells. Postulate interactions of dehydrins with types of surfaces, rather than specific macromolecular classes. K-segment: amphiphilic hydrophobic interaction with partially denatured proteins, membranes; synergistic action with compatible solutes in stabilizing macromolecules and protoplasm; solubilizing agents (deter properties); dehydrin-sugar interaction.* Distribution: nucleus and cytoplasm; nuclear location depends on tissue and dehydrin concerned. Repeating elements postulated to e α helices; Speculated function of both amphiphilic (group 5) is ion sequestration D-7 and D-29. May preserve membrane structure; potential for α helix formation.
3 (III)		D-7	PF004238	11-mer repeating TAQAAKEKAGE	
4 (IV)		D-113	PF03760		
5 (V)		D-29		11-mer repeats; some homology with D-7	Sequesters ions

6 (VI)			α Helical conformation; along with some group 3 members, are the only other group to ha structure in water.
1a	4	GGQTRREQLGEEGY-SQMGRK	
1b	6		
2a	1	DEYGNP (Y domain)	Produced late in embryogenesis; not associated with cold tolerance.
2a	10	EEKK (K domain)	As for 2a
2b	3	S _n (S segt)	Associated with cold tolerance; a fe produced during embryogenesis.
3a	2	TAQAAKEKAGE	
3b	5		
6	7	?	
Members of "conventional" groups 4 and 5 incorporated into LEA groups 2 and 3, according to Wise and Tunnacliffe (2004).			

oligomers of 9 to greater than 30 subunits (Vierling 1991, Mtwisha et al. 2006). According to Wehmeyer and Vierling (2000), expression of *sHSP* ultimately occurs intracellularly in all the embryo tissues during drying, thus suggesting a general protective role in the cells during dehydration and in the dehydrated state. The interaction of small HSPs as molecular chaperones with other proteins has been suggested to counteract inappropriate protein–protein interactions, thus affording this type of general protection in the dry state (reviewed in Buitink et al. 2002) and to facilitate appropriate refolding upon hydration (Mtwisha et al. 2006). In addition to small HSP association with desiccation tolerance, Bettey and Finch-Savage (1998) correlated the quantity of HSP17.6 with longevity in dry *Brassica oleracea* seeds. Seed longevity after priming is generally reduced, and Soeda et al. (2005) showed that the transcripts of two *sHSP* genes—including that coding for HSP17.6—were reduced in osmo-primed *B. oleracea* seeds, which was also a germination-related response. Also relevant are the findings of Yamagishi et al. (2005) that *tan* mutant embryos of *Arabidopsis thaliana*, which contain reduced levels of the RNA encoding HSP17.4, also show compromised desiccation tolerance, among other defects. Interestingly, although HSPs purportedly should counteract the effects of thermal stress, despite the associated expression of *HSP101*, *HSP70*, and *HSP17.6*, both megagametophytes and microgametophytes of *Brassica napus* remained thermosensitive (Young et al. 2004).

While small HSPs have been associated with desiccation tolerance in seeds of a variety of species, they have also been reported to be constitutively expressed in the resurrection plant *Craterostigma plantagineum*, in which they accumulate increasingly during desiccation (Alamillo et al. 1995). Those authors also reported that exogenous abscisic acid (ABA) induced both the expression of the small HSPs and the acquisition of desiccation tolerance in previously desiccation-sensitive callus of *C. plantagineum*. The conjecture that small HSPs are involved in desiccation tolerance is indirectly further supported by coordinated expression of *sHSP* and *lea* transcripts in response to ABA in developing orthodox seeds (reviewed in Buitink et al. 2002). However, as HSP has been reported to be abundant in recalcitrant *Castanea sativa* seeds (Collada et al. 1997), the role of at least some of these proteins in more general stress protection is likely. As an example, Soto et al. (1999) reported that heterologous expression of a plant small HSP in *Escherichia coli* afforded protection against cold and heat stress, suggested by chaperone activity that maintained soluble cytoplasmic proteins in their native state in vitro.

While there is increasing evidence that the small HSPs appear to be implicated in intracellular protection during seed desiccation (e.g., Alpert and Oliver 2002), it is the other class (or possibly, subset) of heat-soluble, hydrophilic proteins—the LEAs—that have been the major investigative focus for the past 25 years.

Attention first became focused on plant LEAs in the 1980s, when it became apparent that several major mRNA species became increasingly abundant in maturing embryos of cotton (*Gossypium hirsutum*) seeds (Galau and Dure 1981). After cDNA-mRNA hybridization studies (Galau and Dure 1981), in vitro and in vivo translation (Dure et al. 1981), and the demonstration that many of the mRNA sequences were specifically increased by the application of exogenous ABA, Galau et al. (1986) coined the terms LEA mRNAs and LEA polypeptides. Since then, however, the nomenclature of the ever-increasing spectrum of LEAs characterized has, in parallel, become increasingly confusing. In 1997, Close, commenting just on the dehydrins (group 2 LEAs), explained that this was the consequence of the “temporary” naming of proteins and clones by the many conditions used in their discovery and that single mnemonics are commonly applied to coordinantly induced families of proteins or cDNAs, although they are immunologically distinct.

As initially we, too, were victims of the confusing nomenclature, we have attempted to illustrate the equivalence of the parallel terminology by reference to a few key older and some more recent publications (Table 6.1). Thus, for example, group 2 (II) LEAs, if categorized by family, constitute the D-11 dehydrins, related on the basis of a common peptide motif (Pfam domain). Because, to date, the group 2 LEAs/dehydrins have been studied more intensively than LEAs of any of the other groups, more is known, or able to be conjectured, about their roles and tissue or cellular distributions. As can be seen from Table 6.1, the distinguishing feature among the LEA groups or families is the characteristic motif (repeated amino acid sequence). Recently, Wise (2003) and Wise and Tunnacliffe (2004) attempted to further refine LEA categorization, resulting in a suggested subdivision of the “traditional” groups 1, 2, and 3, with incorporation of “conventional” groups 4 and 5 into groups 2 and 3 and the creation of superfamilies. The rationale for this recategorization is based on a novel computational approach (the protein or oligonucleotide probability profile [POPP]) using similarities of peptide composition, rather than sequence similarities of proteins.

One of the most lucid reviews of the LEA proteins is that of Cuming (1999), who pointed out that the basis of supposing that the LEAs are involved in tolerance of desiccation comes almost entirely from “correlative and circumstantial evidence, rather than by direct experimental demonstration.” This remains the situation presently, as reflected earlier. However, the basis of the evidence is convincing, in that the appearance and accumulation of LEAs are associated with both orthodox seed maturation and imposition of a variety of stresses that cause water deficits in plant cells, particularly those of the resurrection plants (Cuming 1999). As indicated by that author, *lea* gene expression in response to ABA and jasmonic acid is another unifying feature of the correlation between stress imposition and LEA protein synthesis.

The general characteristics of LEA proteins include the fact that cysteine residues are lacking, that they are composed predominantly of charged and uncharged polar amino acid residues, and that most, with the exception of group 5 LEAs, are highly hydrophilic, facilitating their remaining in solution on heating to boiling or near-boiling temperature (Cumming 1999). In contrast, those of group 5 are considerably less hydrophilic, being possessed of a significantly higher proportion of hydrophobic amino acid residues, and are not heat-tolerant.

Seed Em protein and homologues typifying LEA group 1 have been shown to have a 20-amino acid conserved domain (Table 6.1) that occurs between one and four times (Cumming 1999). Group 1 LEAs (in common with those of group 2) are predicted to have structural flexibility indicated by random coil configuration (McCubbin et al. 1985, Baker et al. 1988) and consequently to become configured to various intracellular surfaces. Because group 1 LEAs are highly hydrophilic, they have a high potential for hydration (McCubbin et al. 1985), which is suggested to be a property shared with group 4 LEAs (Cumming 1999) and possibly, as discussed later, the “ π domains” of group 2 LEA proteins (Close 1997). As a result of both their structural flexibility and water-retaining properties, it has been hypothesized that group 1 LEAs could act to provide a hydration shell around intracellular structures, including macromolecules and membranes, in desiccated tissues of seeds as well as resurrection plants (Cumming 1999, reviewed in Alpert and Oliver 2002, Buitink et al. 2002). Phenotypic analysis of the *Arabidopsis* group 1 LEA, AtEM6 tDNA insertion mutants has shown that the protein plays a role in buffering the rate of dehydration during the later stages of seed maturation (Manfre et al. 2006). Such LEAs are not upregulated in vegetative tissues of desiccation-sensitive *Arabidopsis* in response to any abiotic stress treatment: Their expression in seeds and desiccated vegetative tissues only of resurrection plants (A. Ngubane, N. Illing and J. M. Farrant, unpublished data) suggest that they may play a role specific to desiccation tolerance.

All the group 2 LEA proteins (dehydrins) characterized include between 1 and 11 copies of a lysine-rich K domain or segment, and many contain a tract of serine residues, constituting the S segment, while a third consensus domain, the Y segment, is often present near the amino terminus (Close 1996, 1997) (Table 6.1). Additionally, there are the repeating π segments, which may be tandem repeats; they are highly polar, unstructured regions that have been proposed to interact with, and stabilize, various intracellular constituents (Close 1997, Cumming 1999), and Close (1996) conjectured that hydration of the hydrophilic regions could accordingly result in a surrounding “envelope of ordered water.” Desiccation accompanies cold acclimation and chilling/freezing tolerance in dormant buds. In this regard, Rinne et al. (1999) reported enzyme activity in desiccated birch buds to occur in association with the presence of dehydrins, suggested as a result of the hydrophilic properties of the

latter. The K segment has the propensity to form amphipathic α helices (Close 1996), leading to the possibility of their interaction with, and stabilization of, hydrophobic domains of other proteins that could become exposed with increasing dehydration (Close 1997). Such interactions might counteract inappropriate inter-molecular hydrophobic associations (Cuming 1999), as has been suggested for HSPs (see earlier).

Via their 11-mer repeats, group 3 LEA proteins are also proposed to have the potential for α helix formation. Dure (1993) proposed that dimerization via the hydrophobic stripes at the surfaces of two helices might occur and that the resultant structure could be involved in ion sequestration during dehydration and in the desiccated state. As discussed later, experimental evidence for group 3 LEA coiling upon dehydration has since emerged (Wolkers et al. 2001, Goyal et al. 2003), and Goyal et al. (2005) showed that the nematode group 3 LEA-like protein can itself prevent aggregation of other proteins under desiccated conditions. Although group 5 LEAs differ from those of the other LEA groups in their heat insolubility and considerably higher proportion of hydrophobic residues (Cuming 1999), at least some have been found also to be composed largely of 11-mer repeats (Dure 1993). Representatives of group 5 LEAs (Baker et al. 1988), as well as a protein from *Craterostigma plantagineum* (Piatkowski et al. 1990), share the 11-mer repeating unit, the properties of which are described by Dure (1993) as being essentially identical to those of the group 3 D-7 family. Consequently, they too have been proposed to function for ion sequestration (Table 6.1).

Cuming (1999) suggested that the carboxyl-terminal domains of group 4 LEAs should show functional similarity to the π domains of group 2 LEAs (dehydrins) in terms of their high potential for hydration. Group 4 LEAs additionally are predicted to form amphipathic helical structures similar to those proposed for groups 2 and 3 LEA proteins (Cuming 1999). In this regard, Wise and Tunnacliffe (2004) have proposed a scheme whereby members of LEA groups 4 and 5 have been incorporated into groups 2 and 3, respectively.

As far as localization of the LEAs has been carried out, they appear to be widely distributed intracellularly (Close 1996), although caution must be exercised in the interpretation of observations where aqueous fixation or extraction methods may have been used (Wesley-Smith 2001, Berjak 2006). Although almost entirely based on gene and gene transcript data, the functions proposed for the LEAs of all the groups accord well with the necessity for intracellular stabilization and sequestration of ions that become increasingly concentrated as water is lost during orthodox seed maturation and in the tissues of dehydrating resurrection plants. Also, as discussed later, LEAs may be intrinsic to intracellular vitrification (formation of the glassy state) at low water concentrations in orthodox seeds (Berjak 2006).

As intimated earlier, much of what has been inferred about LEAs and their putative functions is derived from expression of *lea* gene transcripts in relation

to seed development or environmental factors imposing water deficits on vegetative tissues (e.g., Cuming 1999). Polypeptide sequences, deduced by analysis of cloned *lea* cDNA (initially for cotton [Baker et al. 1988]), provided a reference facilitating sequence analysis in other species. There is now a wealth of data in the public domain, allowing sequences derived from gene expression to be matched and so identified, but few studies have targeted the proteins themselves, at least partly because of technical difficulties when working with proteins. Exceptions to this include the isolation and characterization of a heat-soluble protein from *Pisum sativum* seeds (Russouw et al. 1995, 1997) and seeds of a legume, *Acacia erioloba* (Mtwisha et al. 2007). Large-scale proteomic analyses (e.g., Boudet et al. 2006, elaborated later) are starting to appear in the literature. However, as yet, studies serve mainly to identify the presence of stress-induced proteins, and while giving some indication of the post-transcriptional regulation during dehydration, there is currently little demonstration of their *in vivo* role in protection against desiccation.

The work reported by Illing et al. (2005) using cDNA sequencing to compare LEAs in seeds and desiccation-tolerant and -sensitive plant tissues identified 13 upregulated *lea* genes, the transcripts of which were expressed exclusively in the seeds. At least 16 different *lea* genes were found to be expressed during desiccation in leaves of the resurrection plant *Xerophyta humilis* (Collett et al. 2004), including LEA6 (*sensu* Wise 2003), which had been identified as being “seed specific” in *Arabidopsis* (Illing et al. 2005). This evidence, and data pertaining to sucrose accumulation and induction of 1-Cys peroxiredoxin, originally thought to be seed specific (Aalen 1999), has led to the suggestion that desiccation tolerance in vegetative tissues might be a seed-derived trait (Illing et al. 2005). Using real-time quantitative reverse transcription–polymerase chain reaction, Ali-Benali et al. (2005) monitored the appearance of transcripts of five *lea* genes, four from group 2 (dehydrins) and one coding for a group 4 LEA, in wheat. While one of these (*Td11*, coding for an SK₃-type group 2 LEA) was expressed throughout seed development, three others (two group 2 and the one group 4 LEA) appeared during the stages of cell expansion and desiccation, but no transcripts of one gene (*Td25a*) could be detected at any stage during seed development. That work showed that major variations in the pattern of *lea* gene expression and in the relative levels of transcripts accompany wheat seed development (Ali-Benali et al. 2005). Those authors conjecture that the abundant transcripts of *Td11* during the earlier stages of seed development might be indicative of “advance defense” against the onset of dehydration.

From their investigation of wide-range gene expression in relation to acquisition (or reinduction) of desiccation tolerance of *Medicago truncatula* seeds (see later), Buitink et al. (2006) identified a class containing all the *lea* genes, with the exception of two. The former comprise an individual cluster of genes that are upregulated during the reimposition of desiccation tolerance.

Genes coding for 18 LEA proteins (and two HSPs) were among the 187 upregulated genes common to both developmental acquisition of desiccation tolerance and its reestablishment (see later).

One of the few studies in which the broad spectrum of heat-stable proteins (the heat-stable proteome) associated with desiccation tolerance has been studied is that on *Medicago truncatula* (Boudet et al. 2006). Those authors worked on imbibed seeds prior to the loss of desiccation tolerance (prior to radicle emergence) and, to identify LEAs specific to this stage, carried out parallel analyses on desiccation-sensitive germinated seeds at two stages, corresponding to the ability, or not, for the reimposition of tolerance upon incubation in an osmoticum. Boudet et al. (2006) identified a total of 15 polypeptides linked unequivocally with desiccation tolerance in *M. truncatula*, on the basis of their abundance. Among these, six LEA proteins were identified as one from group 1 (MtEm6), one group 2 LEA (one isoform of the dehydrin, DHN3), and three members of group 3 (MP2, described as the basic isoform of PM18; the other two were isoforms of SBP65) and one group 5 LEA (MtPM25). Expression of all the genes concerned, except that coding for the DHN3 isoform, was shown to be seed specific (Boudet et al. 2006). Nevertheless, the authors point out that despite the evidence linking the abundance of the LEA proteins and desiccation tolerance, the causal relationship is difficult to assess and thus must still be assumed. Additionally, the incidence of several other LEAs was shown to be associated with the drought stress imposed by incubation in the osmoticum, these proteins appearing whether or not desiccation tolerance could be reimposed. These proteins were dehydrins (group 2 LEAs) that were expressed as a consequence of drought stress, and Boudet et al. (2006) express the opinion that they are unlikely to be triggered by the same factors as those inducing desiccation tolerance—in agreement with the earlier contention of Black et al. (1999). A further suggestion made by Boudet et al. (2006) is that dehydrins may function for protection only at intermediate hydration levels, rather than at the low water concentrations typifying desiccation tolerance. Those authors further contend that the LEAs identified as being associated with desiccation tolerance might function somewhat differently as water is progressively lost, compared with their putative roles in the desiccated state, and that post-translational modification, such as phosphorylation, could occur, as shown for *Craterostigma plantagineum* proteins during desiccation (Röhrig et al. 2006).

6.3.2 Carbohydrates

In 1977, Amuti and Pollard published information on the soluble carbohydrates of developing and dry seeds, and since then accumulation of nonreducing sugars, notably sucrose with certain oligosaccharides, has been correlated

with desiccation tolerance in seeds (reviewed in Vertucci and Farrant 1995). Yet in the keystone publication *Membranes, Metabolism and Dry Organisms* (Leopold 1986), sucrose was accorded only slight attention as being implicated in the mechanisms by which seeds withstand desiccation: the oligosaccharide raffinose was suggested to be important in prevention of sucrose crystallization at low water concentrations (Leopold and Vertucci 1986) or in stabilizing dry membranes (Crowe and Crowe 1986). In contrast, trehalose was prominently discussed by Crowe and Crowe and a number of other authors (see Leopold 1986) as a disaccharide with a remarkable potential to protect membranes against the adverse effects of virtually complete dehydration and by Burke (1986) in the context of its ability to form an aqueous glass at low water concentrations. However, trehalose is apparently absent in maturing or mature orthodox seeds (Bewley and Black 1994), although surprisingly, its presence has recently been recorded in *Arabidopsis thaliana* seeds (Fait et al. 2006). Since trehalose has been shown to be an “exceptional” stabilizer of proteins (Kaushik and Bhat 2003), Fait et al. (2006) conjecture that its role may be to maintain the conformation of both storage and housekeeping proteins during dehydration in seeds of *A. thaliana*. However, sucrose, which occurs at high concentrations in all desiccated orthodox angiosperm seeds, has widely been considered as an alternate to trehalose—originally in the context of direct membrane protection in the desiccated state as conjectured for trehalose (the “water replacement hypothesis”; e.g., Clegg 1986)—but increasingly in terms of intracellular glass formation (see later).

Accumulation of sucrose and particularly the raffinose series oligosaccharides and galactosyl cyclitols has been recorded to accompany the acquisition of desiccation tolerance and to persist in the dry state in a wide variety of seeds (see e.g., Koster and Leopold 1988, Kuo et al. 1988, Leprince et al. 1990, Blackman et al. 1992, Horbowicz and Obendorf 1994, Steadman et al. 1996, Obendorf 1997, Sinniah et al. 1998, Bailly et al. 2001, and considered in reviews by Buitink et al. 2002 and Kermode and Finch-Savage 2002). Accumulation of these soluble carbohydrates has been linked universally to protection of the intracellular milieu. Localization of soluble carbohydrates within cells of embryonic axes and cotyledons appears to show some variation in seeds across species (Obendorf [1997] and references therein). However, Obendorf (1997) has generalized that, during seed maturation, oligosaccharide synthesis occurs in the “nonparticulate” cytoplasm (cytomatrix).

The pathway of synthesis of the raffinose series oligosaccharides (Obendorf 1997) proceeds via glucose-1-phosphate, which is sequentially converted to UDP-glucose and UDP-galactose. Reaction of UDP-galactose with *myo*-inositol, catalyzed by galactinol synthase, results in the formation of galactinol, after which transfer of the galactosyl residue from galactinol to sucrose forms raffinose in a reaction mediated by raffinose synthase. Stachyose, the other commonly occurring oligosaccharide of the series, is formed by the transfer

of a galactosyl residue from galatinol to raffinose, catalyzed by stachyose synthase. The cytomatrical *myo*-inositol participating in this pathway is derived via glucose-6-phosphate, which, along with glucose-1-phosphate, originates from the breakdown of amylose in the plastids (Obendorf 1997).

A wide-reaching study utilizing transcriptome profiling and analysis for certain metabolites in *Medicago truncatula* (Buitink et al. 2006) have provided valuable pointers about sucrose accumulation during the transition from the desiccation-sensitive to the -tolerant stage. Those authors manipulated germinated seeds that had 3-mm protruded radicles, using PEG incubation to reinstate desiccation tolerance, as well as monitoring developing seeds in relation to the establishment of desiccation tolerance. As a consequence, expression of genes that either were, or were not, common to developmentally related and reimposed desiccation tolerance could be ascertained. During the PEG incubation, sucrose was shown to accumulate first very rapidly (from $15 \text{ mg} \cdot \text{g}^{-1}$ dry mass to $65 \text{ mg} \cdot \text{g}^{-1}$) and then more slowly to approximately $85 \text{ mg} \cdot \text{g}^{-1}$. Profiles of differentially expressed genes in radicles from the PEG-incubated seeds were interpreted as indicating different origins of the sucrose accumulated in the two phases. Initially, the gene coding for β -amylase was upregulated, its expression coinciding with starch degradation. This was later followed by enhanced transcript appearance for starch phosphorylase, which coincided with a second peak of starch degradation (Buitink et al. 2006). Those authors also recorded increased expression of genes coding for enzymes involved in sucrose synthesis, viz. sucrose phosphate synthases and sucrose synthase. As enhanced expression of genes coding for enzymes of β -oxidation also occurred, Buitink et al. (2006) suggested that, under conditions of osmotic stress imposed by the PEG incubation, the main reserves mobilized to produce sucrose are starch and lipid.

A comparison between the data sets for genes involved in PEG-reimposed desiccation tolerance and that which was developmentally-regulated as seeds matured showed commonality of 187 upregulated and 108 downregulated genes. Those genes related to storage reserves were prominent among the upregulated category, while those downregulated were associated with active processes of metabolism (Buitink et al. 2006).

There is no doubt that accumulation of sucrose and, usually, also of certain oligosaccharides is a common feature of the acquisition of desiccation tolerance—in angiosperm seeds, those of gymnosperms (Tersikh et al. 2005), and in the resurrection plants (Illing et al. 2005, Berjak 2006) (see Chapter 3). In this regard, Steadman et al. (1996) indicated that for orthodox seeds of several species, the sucrose-to-sucrosyl-oligosaccharide mass ratio was in the order of 7:1, although this was not invariable. (It should be noted, though, that desiccation-sensitive [recalcitrant] seeds were recorded as having sucrose-to-oligosaccharide ratios of the order of 12:1 [Steadman et al. 1996]. In the context of the suggested continuum of seed postharvest behavior from

“decreasing orthodoxy” through the intermediate to the recalcitrant category [e.g., Pammenter and Berjak 1999], these indicated ratios may possibly be the basis of the statement that seeds with low[er] mass ratios of sucrose to soluble oligosaccharides can be stored longer than those in which the ratio is higher [e.g., Obendorf 1994, Buitink et al. 2002].)

What is the significance to seed desiccation tolerance of the accumulation of sucrose and oligosaccharides? Despite much experimentation and conjecture, this remains a somewhat enigmatic question.

Sucrose accumulates with the onset of desiccation tolerance, but, as noted earlier, desiccation-sensitive recalcitrant seeds across a variety of species appear to accumulate even more of this disaccharide relative to oligosaccharide (Steadman et al. 1996). However, seeds of the latter category lose viability at water concentrations significantly higher than those at which sucrose will contribute to intracellular vitrification or any other type of membrane-stabilizing interaction (Pammenter and Berjak 1999). This, however, does not gainsay the importance of sucrose, particularly in terms of intracellular vitrification (see later), in orthodox seeds during maturation drying and in the dehydrated state. Rather, in desiccation-sensitive, recalcitrant seeds, high sucrose concentrations should be considered in the context that metabolism is ongoing, grading imperceptibly from developmental to germinative events. At all stages, a ready source of respiratory substrate is required—and especially when germination and seedling establishment occur. This requirement would be satisfied—and can be adequately explained—by high intracellular sucrose availability.

In terms of the oligosaccharides, Black et al. (1999) found that the induction of desiccation tolerance was not correlated with raffinose accumulation in wheat embryos. However, Bochicchio et al. (2000) reported reduced storage potential to be consistently associated with lower raffinose content of experimentally manipulated maize embryos. The occurrence of the oligosaccharides also does not appear to be intrinsic to cytoplasmic glass stability. Buitink et al. (2000) showed that while osmopriming of *Impatiens walleriana* seeds lowered the oligosaccharide content and concomitantly increased sucrose levels, there was no accompanying difference in the glass transition temperature (T_g). However, despite the apparent stability of the glassy state (see later), those authors reported that seed storage life span was significantly reduced in seeds dried back and maintained at low RH after priming.

It therefore seems that the oligosaccharides that are accumulated on desiccation must have a role(s) different from that so far favored, viz. the prevention of sucrose crystallization as water concentrations decline during orthodox seed maturation drying. As suggested by Buitink et al. (2002), while oligosaccharides fulfil this role in model sugar glasses (Caffrey et al. 1988), the many nonsugar components of the cytomatrix would probably preclude sucrose crystallization.

As indicated earlier, the presence of oligosaccharides in seed embryos appears to be correlated with storage stability. It is possible that the oligosaccharides could be a byproduct formed as respiratory substrates are removed from the dehydrating cells. Considering that raffinose and stachyose are galactosyl oligomers and that the pathway for their synthesis originates with glucose-1-phosphate and glucose-6-phosphate (Obendorf 1997), we suggest that the abstraction of the respiratory substrates from the cytomatrix may be at least partly affected by their incorporation into the oligosaccharides. The decline in respiratory substrates in developing seeds was recorded in 1977 by Rogerson and Matthews, and the recent evidence of Buitink et al. (2006) offers the explanation that some of the genes repressed in response to declining water content are those controlling primary and energy metabolism. In addition to this metabolic switch-off, monosaccharides already present and/or still being slowly liberated could be removed by incorporation into the oligosaccharides and, as suggested by Berjak (2006), into sucrose.

There has been much debate about the ability of sucrose to confer membrane stability in the dry state of seeds and pollen, by direct interaction with the polar lipid headgroups (the “water replacement hypothesis”), and, although direct evidence is generally lacking (Hoekstra et al. 1997), this idea remains entrenched in the literature (e.g., Hoekstra et al. 2001, Buitink et al. 2002, Boudet et al. 2006). The introduction, and persistence, of the concept of sucrose taking the place of water on lipid headgroups and so suggested as contributing to the maintenance of phospholipid spacing in membranes, thus lowering the gel transition temperature (T_m), substantially derive originally from the many studies in which trehalose has been similarly implicated (e.g., Clegg 1986, Crowe et al. 1987, 1996). There is no doubt that trehalose is remarkably effective in preserving the integrity of phospholipid vesicles, but much of the evidence for this has been obtained from liposomes or isolated membranes after freeze-drying (see, e.g., Crowe et al. 1996 and Hoekstra et al. 1997, and references therein) or air-drying (Ricker et al. 2003). There is, however, evidence for remarkable desiccation tolerance in organisms that naturally contain trehalose, such as *Artemia* (brine shrimp) cysts (Clegg 1986) and the yeast *Saccharomyces cerevisiae* (Leslie et al. 1994), and bacteria have been shown to tolerate freeze-drying better in the presence of trehalose (Leslie et al. 1995). However, in 1996, Crowe et al. mentioned that both direct interaction of the sugar and lipid headgroup and glass formation are often necessary for stabilization, and, in 2002, Crowe et al. expressed the view that additional means of stabilizing dry systems are almost certainly required.

While a special case may be made for trehalose compared with other sugars for liposome or membrane stabilization in the dry state (Crowe et al. 1996), and trehalose has been described as being an exceptional protein stabilizer (Kaushik and Bhat 2003), it is apparently not present in angiosperm seeds, the only recorded exception being those of *Arabidopsis* reported by Fait et al.

(2006). The sucrose content of maturing and dry orthodox seeds generally is high, which focused attention on this disaccharide as possibly fulfilling the role in water replacement that had been emphasized for trehalose in desiccation tolerance. Hoekstra et al. (1992) related desiccation tolerance in pollen to the presence of sucrose, with the rider that a high degree of phospholipid fatty acid unsaturation might also confer protection. Sun et al. (1996) examined the responses of liposomes air-dried in the presence of sucrose and found them to exhibit increased stability in the glassy state. The opinion that sucrose might act in water replacement in a manner analogous to trehalose has been reiterated, although Crowe et al. (1996) had pointed out the superior properties of trehalose over sucrose, at least in model liposome systems. Also, because interaction between sucrose and the phosphate of membrane phospholipids could not be clearly established, Hoekstra et al. (1997) suggested that factors additional to sucrose might be required to confer membrane stability. In that publication, Hoekstra et al. proposed the possibility of amphipathic compounds, such as flavinols, conferring membrane protection in the dry state in desiccation tolerance in plants, a view for which evidence has since accumulated (Hoekstra et al. 2001, Golovina and Hoekstra 2002).

It is not out of the question that sucrose might interact directly with the lipid headgroups at extremely low water contents—or in the virtual absence of water ($<0.02 \text{ g} \cdot \text{g}^{-1}$) (Koster and Bryant 2005). However, we are of the opinion that too much reliance for this occurring in dry seeds (or pollen) has been placed on the outcomes of experiments with lyophilized model liposomes, and especially in drawing parallels with what has been proposed for trehalose.

As reviewed by Koster and Bryant (2005), as water is withdrawn through intermediate levels (Ψ from -6 to -60 MPa), cell membranes move closer together. It is then that hydration forces, which are repulsive forces counteracting the close approach of opposing hydrophilic surfaces, become increasingly operative. However, concomitantly, if membrane surfaces come to be positioned sufficiently close to one another, lateral compression can result, with the possible consequence of transition of some phospholipids to the gel phase, and even demixing of membrane components and exclusion of integral proteins. Sugars within the aqueous phase between opposing membrane surfaces will help to counteract the close approach of the membranes, acting physically as volumetric and osmotic spacers (Koster and Bryant 2005). Consequently, compressive stress in the lipid bilayer is limited (Bryant et al. 2001). This proposed protective mechanism is described as being nonspecific, as long as the molecular volumes of the sugars concerned are not large enough to result in their being displaced from intermembrane spaces into discrete bulk phases during dehydration (Koster et al. 2000 2003). At low hydration levels ($\Psi < -60$ MPa), sugars will contribute toward the glassy state (see later): in the context of membrane stability, glasses exert a tensile stress on membranes,

thus facilitating resistance to phase transition and a lower phase transition temperature (T_m) (Koster and Bryant 2005). Recent work on protoplasts prepared from germinated pea embryos in which desiccation sensitivity had been reinstated indicated that, on subsequent dehydration, elevated intracellular sucrose concentration decreased the incidence of membrane damage (Halperin and Koster 2006). As orthodox seeds will seldom naturally attain water contents as low as $0.02 \text{ g} \cdot \text{g}^{-1}$ when sugars may well become hydrogen-bonded to the polar surfaces of membrane lipids, from biophysical data it seems far more likely that the role of sucrose is dynamic—in hindering the close approach of membranes to one another (Bryant et al. 2001, Koster and Bryant 2005, Halperin and Koster 2006).

6.3.3 *The Basis of Glass Formation*

Evidence for the existence of the intracellular milieu in the glassy (vitrified) state in desiccation-tolerant seeds and other plant tissues has gained considerable support since it was first proposed in the late 1980s (by, e.g., Koster and Leopold 1988, Williams and Leopold 1989, Koster 1991). However, there has been an increasing realisation that although sugars must contribute to the formation and persistence of intracellular glasses in dry seeds, these cannot be basically composed of supersaturated sugar solutions alone (see later). A glass describes a metastable amorphous solid not having a regular structure, as opposed to the crystalline state, which is a solid with regular structure. To appreciate this, the reader is referred to the article by Walters (1998). The glassy (or vitrified) state results as increasing dehydration causes solutions to become supersaturated, and hence highly viscous, as does cooling of a solution below the T_g , as long as the crystalline phase is avoided (Burke 1986). Upon dehydration *in vitro*, sugar solutions will become supersaturated and have the propensity to form glasses under appropriate conditions, and it was Burke (1986) who suggested that vitrification may contribute to life in the dry state. During the late 1980s and into the 1990s, conjecture about, and evidence that, glass formation occurs in orthodox seeds during maturation drying was forthcoming, generally based on the presence of high intracellular sucrose concentrations in appropriate mass ratios with raffinose series oligosaccharides or galactosyl cyclitols (e.g., Koster and Leopold 1988, Williams and Leopold 1989, Koster 1991, Leopold et al. 1994, Obendorf 1997). The glassy state in dry seeds must impose highly restricted molecular mobility—or the mobility of damaging free radicals (reviewed by Berjak 2006)—and is also held to confer stability on macromolecules and membranes (reviewed by Leopold et al. 1994). Hence, formation of intracellular glasses is entirely compatible with survival of orthodox seeds in the dry state.

However, even early on, doubts were expressed about the possibility of intracellular glasses being simply sugar glasses: for example, experiments conducted by Koster (1991) showed that in model systems constituted to simulate intracellular sugar mixtures, there were discrepancies in T_g when compared with those obtained for seeds. Those observations suggested that components additional to the sugars are involved in intracellular glass formation. This was cogently expressed by Walters (1998), who emphasized that the glassy state must involve the constituents of the entire aqueous phase of the cell, not only a few solutes.

6.3.4 Could Certain LEAs Be Major Elements in Intracellular Glasses?

As noted earlier and in Table 6.1, LEAs of certain groups have the potential to form α helices and, although generally considered to be natively unfolded in the hydrated state (e.g., Wise and Tunnacliffe 2004), show varying degrees of order, particularly in terms of the occurrence of β sheets (Boudet et al. 2006). However, it is the demonstrated response for α helix formation upon dehydration—and particularly in the presence of sucrose (Wolkers et al. 2001)—that suggests the implication of at least some of the LEAs in intracellular glass formation (Berjak 2006). Wolkers et al. (2001) characterized a D-7 (group 3) LEA occurring in substantial quantities in pollen of *Typha latifolia*. The protein, which showed highly unordered, random coil conformation in solution, underwent a reversible conformational change to form both α helices and extended intermolecular β sheets on slow drying but formed only α helices when dried in vitro in the presence of sucrose (Wolkers et al. 2001). Those authors postulated that this would also be the conformation adopted by the D-7 LEA within the dry pollen grains, which contain 23% by weight of sucrose. Their findings also led Wolkers et al. (2001) to question the postulated role of the 11-mer repeat of D-7 LEAs in ion sequestration (Table 6.1), as the α helical conformation resulted after dehydration: however, a *caveat* mentioned by those authors was that the investigation did not reveal when during dehydration the protein assumed α helical conformation. A further significant finding from the work on the *T. latifolia* D-7 LEA was that its presence in a sucrose glass increased the T_g and the average strength of hydrogen bonding in comparison with these properties in a pure sucrose glass. This led Wolkers et al. (2001) to suggest that the protein and sucrose may act synergistically in glass formation. Other work has also shown that a group 3 “LEA-like” protein (from a desiccation-tolerant nematode, *Aphelenchus avenae*), which is natively unfolded, will assume a significant α helical component upon dehydration (Goyal et al. 2003). While both the *T. latifolia* and *A. avenae* proteins are group 3 LEAs, a group 1 and a group 5 LEA from *Medicago truncatula* seeds showed a transition to a far more ordered conformation upon dehydration,

leading to the suggestion that this may be a characteristic of all LEA proteins (Boudet et al. 2006).

Oliver et al. (2001) suggested that it is the *de novo* synthesis of LEAs, rather than oligosaccharides, that underlies the intracellular stability of dry, orthodox seeds. However, this suggestion must be viewed in the light of the reality that dehydrated orthodox seeds have high sucrose contents and, seemingly, uniformity in sucrose-to-oligosaccharide mass ratio (Steadman et al. 1996). From the evidence (Black et al. 1999, Bochicchio et al. 2000, Buitink et al. 2000) discussed earlier, the role of the oligosaccharides in intracellular glasses remains equivocal. However, while Buitink et al. (2000) found that priming diminished the oligosaccharide component of dried-back seeds, they also reported that the stability of the intracellular glass was not affected, although it should be noted that the sucrose content increased following priming.

Notwithstanding the range of miscellaneous intracellular components that must inevitably be incorporated into intracellular glasses in dry orthodox seeds, the two universal components appear to be sucrose and LEA proteins. From their work on the nematode “LEA-like” protein, Goyal et al. (2003) have speculated that, upon dehydration, it could form coiled-coils similar in morphology to the flexible cytoskeletal intermediate filaments in animal cells, which confer both strength and resilience. They further conjectured a synergistic association between the coiled LEA protein filaments and sugars within intracellular glasses, with the filaments conferring tensile strength on an amorphous sugar glass.

This idea has been taken further by Berjak (2006), who, on consideration of the evidence, proposed that the intracellular glass in dry seeds is most likely to be based on coiled LEAs in interaction with sucrose and the residual water but that LEAs would be excluded from narrow intermembrane spaces, where essentially sugar-based glass would prevail, as suggested by Bryant et al. (2001). Furthermore, there is evidence that the glassy state may not be uniform and (as discussed earlier) that localized water pools or pockets permitting limited metabolic (or, ultimately, deleterious) reactions exist both in the cytomatrical domain and within organelles and the nucleus (Berjak 2006). Based on her model of the cytoplasmic glass, that author has suggested that in cases where reducing water concentration within Level I (Fig. 6.1) is associated with reduced seed viability (noting that this is common, but apparently not universal), the integrity of intracellular glasses may be compromised. On the basis that the residual water is held to play a critical part in maintaining the glass by hydrogen bonding between LEAs and sucrose, removal of water below a threshold could perturb the structural continuity of the glass (Berjak 2006) and the integrity of macromolecules, rendering them more vulnerable to attack by, for example, free radicals (Walters 1998). This aspect will be considered further, later.

6.3.5 *Reactive (Active) Oxygen Species and the Vital Role of Antioxidants*

From a superficial consideration, one of the most paradoxical paradigms to have emerged recently is the dual role of reactive oxygen species (ROS) in intracellular signaling as well as intracellular destruction. Yet, accumulating evidence supports both these apparently conflicting roles (reviewed in Laloi et al. 2004, Foyer and Noctor 2005, Suzuki and Mittler 2006). ROS are formed when high-energy-state electrons are transferred to molecular oxygen (O_2): ROS include 1O_2 (singlet oxygen), H_2O_2 (hydrogen peroxide), $O_2^{\bullet-}$ (the superoxide radical), and OH^{\bullet} (the hydroxyl radical), which have long been considered as toxic species that can cause oxidative damage to lipids, proteins, and nucleic acids (e.g., Halliwell 1987, Hendry 1993, Fridovich 1998, Suzuki and Mittler 2006). Consequently, activity of the spectrum of enzymatic and nonenzymatic antioxidants has generally been considered as vital in the context of inactivating the ROS—that is, in intracellular protection.

However, in metabolically-active, hydrated plant tissues, ROS are now considered to act as second messengers in a diversity of signal transduction cascades (Foyer and Noctor 2005 and references therein), with H_2O_2 and $O_2^{\bullet-}$ being singled out because of their implication in many plant developmental and growth processes. Foreman et al. (2003) linked the production of OH^{\bullet} generated by the activity of an NADPH oxidase to activation of plasmalemma Ca^{2+} channels, facilitating an influx of this cation and subsequent modulation of growth processes, including actin dynamics, required for root hair growth. In an overview, Noctor (2006) has detailed the interactions of Ca^{2+} signaling and redox metabolites in plant growth and development. In *Arabidopsis*, ROS occurrence and levels have been found to be a function of a network of at least 152 genes (Mittler et al. 2004). Nevertheless, in view of the potentially highly destructive consequences of excessive ROS accumulation, their levels must be strictly controlled; thus, antioxidants themselves (see later) are considered to determine the transmission and specificity of ROS signaling (Foyer and Noctor 2005). In this context, the low-molecular-weight antioxidants also are considered to be involved in signaling. According to Foyer and Noctor (2005), signal transduction cascades in which ROS are involved govern mechanisms as diverse as mitosis, plant development and responses (including tropisms), and programmed cell death (PCD), which includes one route of defense. Consequently, those authors propose that the ROS–antioxidant relationship be described in terms of “oxidative signaling,” rather than only in the framework of damage (“oxidative stress”), as has been the conceptualisation until recently. In this regard, Van Breusegem and Dat (2006) stress that it is important to discriminate between PCD, as the consequence of one or more orderly processes that are genetically controlled, albeit activated by ROS, and necrosis, resulting from phytotoxic accumulations of substances, which could include hyperaccumulation of ROS.

In the context of ROS and antioxidants, what is the situation during orthodox seed development? From work with *Arabidopsis* tDNA insertion mutants, the data of Cairns et al. (2006) show that the protective effects of even a low level of autonomously synthesized glutathione (GSH) is essential for embryo development and seed maturation. In developing wheat seeds, the redox state of the ascorbate and GSH pools was shown to shift toward the oxidised forms during maturation, indicating that the redox balance between the two pairs could affect the activation or inactivation of particular metabolic pathways (De Gara et al. 2003). Those authors have shown that high activity levels of catalase (CAT) and the ascorbate and glutathione redox enzymes characterize the initiation of maturation drying, after which the activities decline, and Bailly (2004) has associated CAT and glutathione reductase (GR) activity with the desiccation-tolerant condition of seeds. From their data, De Gara et al. (2003) suggest that the redox state of the two pairs, together with the protein sulfdryl to disulfide transition, link these factors to both protein maturation and protection against ROS. Subsequently, an *h*-type thioredoxin (reduced by NADPH via the action of NADP-thioredoxin reductase) reduces disulfide bonds of target proteins and has been proposed to act as a germination trigger (Yano et al. 2001).

Most of the work that has been done on developing seeds has been focused on PCD in the endosperm, although several of the studies were aimed at hormonal control. However, it appears that ROS are implicitly—or explicitly—involved. In maize, a balance between ABA and ethylene has been suggested to initiate the onset and progression of PCD during endosperm development (Young and Gallie 2000). Although ABA itself is implicated in a variety of processes in the growing plant and in seeds, its appearance is notably associated with a variety of abiotic stresses, including water deficiency, on the basis that the stress initiates a Ca^{2+} -dependent phospho-relay cascade (reviewed in Xiong and Zhu 2003). In this regard, Ca^{2+} and ABA (with ethylene and salicylic acid) have been shown to afford protection against heat-stress oxidative damage, including enhancement of the activities of different ROS-scavenging enzymes (Suzuki and Mittler 2006, and references therein). While not abiotic, the onset of dehydration in developing seeds could well act as a trigger in a similar way.

Gene expression analyses in developing barley caryopses indicate that while ABA influences the acquisition of desiccation tolerance, PCD in the endosperm appears to be mediated via the ethylene pathway (Sreenivasulu et al. 2006). Interestingly, gibberellic acid (GA) has been implicated in promoting PCD in the lateral regions of the endosperm of tomato seeds, whereas micropylar endosperm cells undergo GA-independent PCD (DeBono and Greenwood 2006). GA was also found to be involved in death of cereal aleurone cell layers; however, application of ABA was found either to prevent, or to delay, aleurone cell death (Fath et al. 2001). Nevertheless, those authors

make the point that PCD does not occur in the aleurone, unlike the case for cells of the endosperm that accumulate storage reserves. However, as aleurone cell deterioration in response to GA treatment was accompanied by declining levels of a variety of enzymatic antioxidants and their transcripts, Fath et al. (2001, 2002) proposed that death was the consequence of the accumulation of ROS.

There is no doubt that stress is imposed on the tissues as orthodox seeds lose water during the final stage of maturation, and, at Ψ at or less than -3 Mpa (water concentration $\leq 0.45 \text{ g} \cdot \text{g}^{-1}$), unbalanced metabolism is likely to occur (Fig. 6.1 [Vertucci and Farrant 1995, Berjak 2006]). Under these conditions, respiratory activity can initially be measured—which may be a function of the mitochondrial matrix representing a localized pool of higher water activity/water concentration (reviewed by Berjak 2006), but other metabolic processes, such as, protein synthesis and repair, are not operative (Vertucci and Farrant 1995). This situation is parallel with that described for heat-stressed vegetative tissues by Suzuki and Mittler (2006) and, unless the strictest intracellular control is exerted, ROS will be generated and accumulate. Although details of the mode of action of many of the protective processes entrained in cells of desiccation-tolerant tissues during dehydration remain speculative (e.g., Pammenter and Berjak 1999, Berjak and Pammenter 2004), a spectrum of antioxidants appropriate to the various hydration levels (Fig. 6.1) must be operative. Additionally, the situation will be ameliorated, the shorter the period at which the seed tissues remain in the danger zone, particularly at water contents less than $0.45 \text{ g} \cdot \text{g}^{-1}$. However, the natural period over which seeds undergo the entire process of maturation drying cannot be curtailed to the point that synthesis and accumulation of protectants, and intracellular modifications (e.g., organelle dedifferentiation), are compromised. It is equally important that the balance between ROS generation and antioxidant activity be maintained when the dry seeds take up water during imbibition. Thus, for example, H_2O_2 levels, which were found to be high in the maize scutellum during the early phases following imbibition, declined thereafter in association with increasing levels of CAT activity (Hite et al. 1999). High activity of CAT also appears to be important as water is lost during the final stages of development in seeds of sunflower and other species (Bailly 2004).

Changes in the spectrum of antioxidants accompanying seed development were reviewed by Bailly (2004), who generalized that, while nonenzymatic antioxidants are likely to confer protection in the dry state, high levels of superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity characterize developing seeds that are still desiccation sensitive, when CAT and GR activities are low. This situation appears to be reversed in the desiccation-tolerant seed condition, when the activities of SOD and APX decline but high CAT and GR activity occurs (Bailly 2004). It is likely that these changes may be linked to ROS signaling that must surely accompany seed development, the

acquisition of desiccation tolerance, and resilience to water loss. This notwithstanding, the significant degree of dehydration accompanying seed maturation (Fig. 6.1) is considered to be a process with the potential to impose considerable oxidative stress (Serrato and Cejudo 2003, Bailly 2004) and it is implicit that the changing antioxidant machinery is as involved in protection, as in modulating ROS signaling.

What is the situation in the desiccated, orthodox seed, when overall water concentration is generally $\leq 0.1 \text{ g} \cdot \text{g}^{-1}$? Production and accumulation of AOS are generally considered not only to be ongoing but also to be a major factor in deterioration of dry seeds (e.g., Hendry 1993, reviewed in Smirnov 1993, Bailly 2004, Berjak 2006). As a counteractive measure, ROS scavenging, suggested by nonenzymatic antioxidants, is necessary (Bailly 2004). In this regard, the data of Sattler et al. (2004) indicate that the primary function of tocopherols in dry seeds is the limitation of nonenzymatic lipid oxidation, and Kranter and Birtić (2005) have demonstrated that the GSH redox potential ($E_{\text{GSSH:2GSH}}$) changes, becoming more positive, as viability is lost in both resurrection plant tissues and seeds held in the desiccated state.

Relaxation of intracellular glasses in dry seeds appears to occur with time and/or at water concentrations less than an optimally low value (Walters 1998, Berjak 2006), and molecular mobility increases with declining cytoplasmic viscosity observed during seed aging (reviewed in Buitink and Leprince 2004). These phenomena must also increase the potential for migration of ROS (Berjak 2006). In this regard, Walters et al. (2004) concluded that even at temperatures lower than -130°C , deteriorative reactions are possible on the basis of the potential for intracellular molecular mobility. What then is the possibility that, within limits, antioxidant mechanisms can operate within dry seeds?

Methods to analyze the situation in desiccated tissues are extremely limited, primarily because most analyses require aqueous conditions. However, use of microscopical methods employing immunocytochemistry on freeze-substituted specimens offers a realistic alternative, as has been shown by Stacey et al. (1999), who localized 1-cys peroxiredoxin to nuclei of barley embryo and aleurone cells.

The peroxiredoxins, which have one or two conserved cysteine residues and no tightly bound metal ions, are thiol-containing antioxidants that function to reduce H_2O_2 and $\bullet\text{OH}$ (Aalen 1999). Besides being identified in barley seeds, 1-cys peroxiredoxin has been shown to be abundantly expressed in desiccating tissues of the resurrection plants, *Xerophyta humilis* and *X. viscosa* (Illing et al. 2005). However, for 1-cys peroxiredoxin—or any other antioxidant, whether enzymatic or nonenzymatic—to be active in desiccated tissues, demands the occurrence of localized regions of sufficiently high hydration (“pockets” of water [see earlier]) that will not only facilitate their reactivity but also ensure their regeneration (Berjak 2006). In the case of the catalytic

center of 1-cys peroxidiredoxin, regeneration of the cysteinyl residue is required, which is normally achieved by intramolecular or intermolecular thiol-disulfide reactions, and ultimately by electron donors such as thioredoxins and glutaredoxins (Dietz 2003). While direct correlative evidence is not available, it may be significant that an *h*-type thioredoxin has been localized to nuclei of aleurone and scutellum of mature wheat seeds (Serrato and Cejudo 2003), coinciding with the occurrence of 1-cys peroxidiredoxin in similar tissues of barley seeds.

It is also feasible that the milieu of the chromatin represents an intracellular site where the hydration level is sufficiently high to permit a measure of organised reactivity. Certainly, exonuclease and endonuclease activity has been reported to occur in desiccated rye seeds (Elder et al. 1987), arguing that even if deteriorative in nature, localized enzyme action can occur. Dehydrins have been localized to nuclei of aleurone and scutellar cells in mature maize caryopses (Ashgar et al. 1994, Close 1996), and Rinne et al. (1999) conjectured that enzyme activity was maintained in dehydrin-associated pools of water in otherwise desiccated birch buds.

Obviously, specific approaches need to be devised, and much work is required, to test the conjectures made earlier.

6.4 Control of Desiccation Tolerance in Seeds

Our understanding of the control of desiccation tolerance, from signal perception and transduction, through the regulatory genes involved, to the controls (pre and post) implicated in transcription and translation, is fragmentary at best. We present here only a cursory overview, and for more detailed reviews of gene regulation of seed development (of which desiccation tolerance forms only a part), the reader is referred to reviews (and references within) by Vicente-Carbajosa and Carboner (2005) and To et al. (2006); of desiccation and drought tolerance desiccation tolerance in general, see reviews by Ramanjulu and Bartels (2002), Bartels et al. (2006), and Bartels et al. (Chapter 5); and for specific aspects on transcription and translation control, see articles by, for example, Cuming (1999), Boudet et al. (2006), Buitink et al. (2006), Fait et al. (2006), and Gutierrez et al. (2006).

Desiccation tolerance is a preprogrammed, developmentally-regulated event in seeds and is initiated by maternal factors rather than environmental signals (Bewley and Black 1994). It forms part of the continuum of events initiated by fertilization of the ovule, proceeding through histodifferentiation and growth (or early embryo morphogenesis [E.E.M.] in Fig. 6.2) to seed maturation. Although desiccation tolerance is initiated during the mid to late stages of maturation, its overlap with maturation processes and the development of

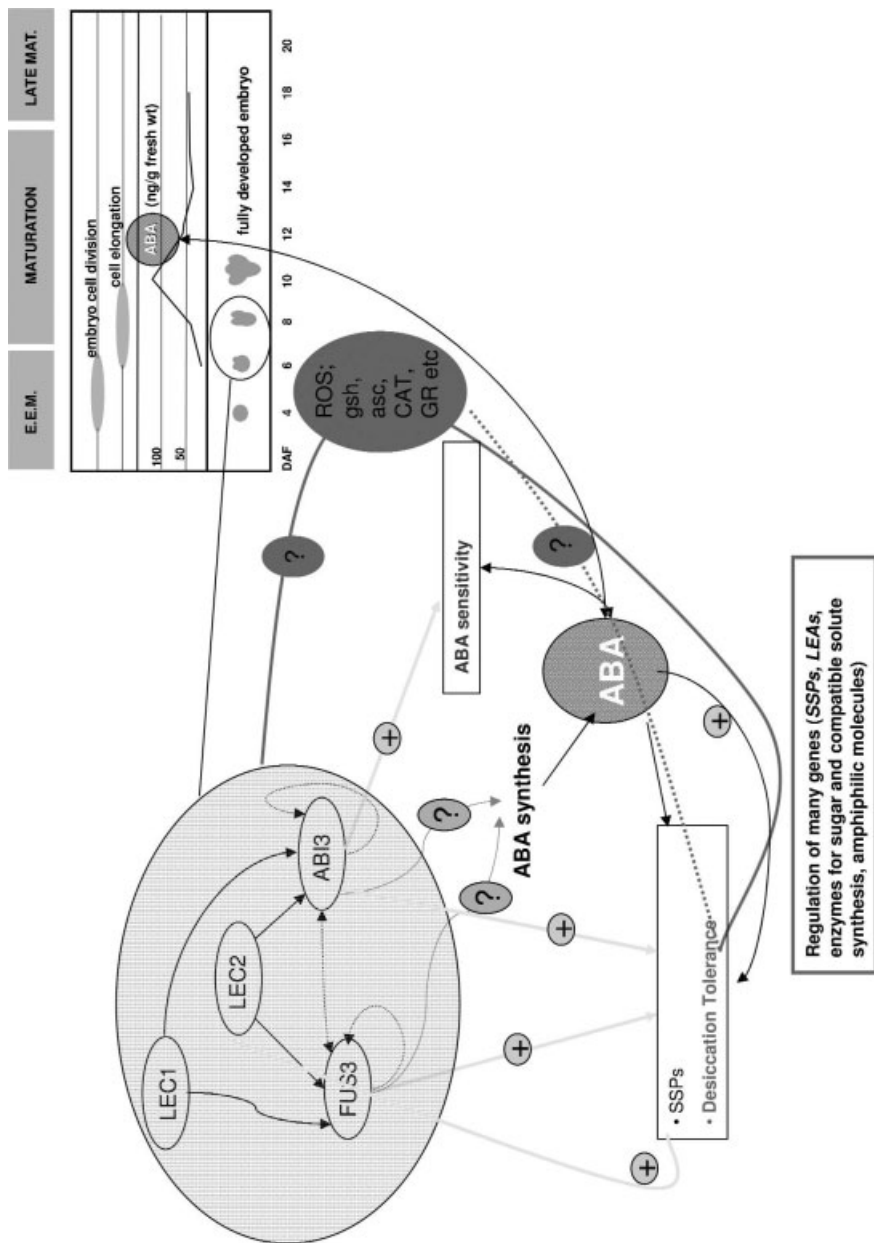
dormancy in many species has complicated our understanding of its control. The onset of desiccation tolerance varies among species from being just prior to, or coincident with, the onset of water loss from the embryonic tissues brought about by the loss of vascular supply of water and assimilates from maternal tissues (Galau and Dure 1981, Greenwood and Bewley 1982, Hughes and Galau 1989), and thus while the process is presumably initiated by parental signals, the ultimate control of desiccation tolerance probably resides in the embryo (and endosperm in monocots) itself.

It has been known for some time that desiccation terminates the processes associated with development and switches on the program for germination in nondormant species (reviewed in Kermode 1990, 1995, Kermode and Finch-Savage 2002). Desiccation tolerance is lost with the onset of germination, usually once radical protrusion through the covering structures has occurred, but there is some plasticity to this switch because desiccation tolerance can be reestablished to a greater or lesser degree prior to this stage of germination by controlled re-drying of the seedlings (e.g., Corbineau et al. 2004, Farrant et al. 2004) or by their treatment with an osmoticum (Buitink et al. 2003, 2006).

Interestingly, the orthodox seeds of resurrection plants such as *Craterostigma wilmsii* also go through the stages of loss of desiccation tolerance during germination and in seedlings prior to its reestablishment in vegetative tissues of mature plants (J. M. Farrant, unpublished data). Thus, although many of the mechanisms of desiccation tolerance might be common to seeds and vegetative tissues of resurrection plants, the control processes involved in their induction might differ.

The initial studies involved in elucidation of developmental programs controlling desiccation tolerance in seeds have come from the isolation and analysis of viviparous mutants and mutants impaired in maturation processes in maize (the *VP* series mutants) and *Arabidopsis* (*LEC1*, *LEC2*, *FUS3*, and *ABI3*). From these studies, and also from those on control of desiccation tolerance in vegetative tissues, it is also apparent that there are ABA-dependent and ABA-independent pathways of gene regulation (reviewed in Bray 1993, Kermode 1990, 1995, Kermode and Finch-Savage 2002, Bartels 2005, Vicente-Carbajosa and Carbonero 2005).

Initially, it was believed that the control of gene expression of maturation and desiccation tolerance was different in monocots and dicots, but the recent review by Vicente-Carbajosa and Carbonero (2005) indicated that there is considerable commonality, especially, for example, in control of *SSP* (seed storage protein) and *lea* genes. Those authors show that there are common gene promoter *cis*-acting motifs and that interacting transcription factors are functionally exchangeable between monocots and dicots and suggest that this indicates a conservation of gene expression and the possibility of a common ancestor. Fig. 6.2 summarizes the current understanding of control of seed



maturation and desiccation tolerance by the four “master genes” and by ABA in *Arabidopsis thaliana*. The diagram also incorporates the potential role(s) of ROS and antioxidants as signals for desiccation tolerance. We envisage that the latter controls come into play only in the late stages of maturation, after loss of vascular connection from the parent, and thus emanate from within the seed tissues themselves. Because their potential role in desiccation tolerance is elaborated in the section on antioxidants, their regulatory influences are not discussed further here.

6.4.1 Absciscic Acid–Regulated Control

In most seeds, the early and mid phases of maturation are controlled largely by the action of ABA, which is initially synthesized in maternal tissues but in later development within the embryo and endosperm (Nambara and Marion-Poll 2003). ABA controls transcription of the *SSP* genes, and possibly the genes responsible for accumulation of other reserves (Quatrano 1987, Vicente-Carbajosa and Carbonero 2005). High levels of ABA, or appropriate ABA/GA levels, are thought to maintain the seed in a quiescent or dormant state, germination being brought about by the reduction in ABA and/or reversal of ABA/GA ratios (Karssen et al. 1983, Quatrano 1987, White and Rivin 2000, Vicente-Carbajosa and Carbonero 2005). Thus it is not surprising that ABA is thought to play an important role in acquisition and loss of desiccation tolerance.

Probably the best reported role for ABA in control of desiccation tolerance is its role in regulation of *lea* gene transcription (reviewed in Bray 1993, Kermode 1990, 1995, Cuming 1999, Kermode and Finch-Savage 2002). In many cases, exogenous application of ABA can stimulate the accumulation of LEAs in seeds (Cuming 1999, Kermode and Finch-Savage 2002) and resurrection plants (Ingram and Bartels 1996).

Fig. 6.2 (Left) Stages of development (early embryo morphogenesis, EEM, and maturation, MAT) and ABA accumulation in seeds of *Arabidopsis thaliana* are shown at the *top right* (adapted from Vicente-Carbajosa and Carbonero 2005). A summary of the master gene control of maturation events, which include the acquisition of desiccation tolerance, within the embryo (from the heart-stage onward) are given in the *gray oval* on the left. Control networks among the master genes are depicted by *black lines*; *solid lines* depict control of one locus over another; and *dashed lines* depict regulatory influences among them and upon themselves. Downstream actions of the genes are given as *yellow lines*. Control via ABA and ROS are shown in *blue* and *pink ovals*, respectively. Unknown pathways are represented by question marks. LEC = Leafy cotyledon; FUS3 also belongs to the leafy cotyledon phenotype, usually with anthocyanins and trichomes; ABI3, abscisic acid insensitive; gsh, glutathione; asc, ascorbate; CAT, catalase; GR, glutathione reductase; SSPs, seed storage proteins. For color detail, please see color plate section.

The first understanding of ABA regulation of *LEA* genes in seeds came from studies on the wheat *Em* gene (a group 1 *LEA*, Marcotte et al. 1989) and the *RAB* (responsive to ABA) genes, such as *RAB16*, a group 2 *LEA*/dehydrin (Mundy et al. 1990). These *LEAs* contain (like all other *LEAs* that are regulated by ABA) the ABA-responsive sequence element (ABRE) that has the conserved core sequence ACGT. This motif is the core of the “G-box”—CCACGTGG—found to occur in a large number of plant promoters. In these (and many other) *LEAs*, transcription is promoted through interaction with b-ZIP DNA-binding proteins (Guiltinan et al. 1990). What has become clear is that while all ABA-regulated genes contain G-boxes, not all G-box elements necessarily confer ABA inducibility *in vivo*, and thus the G-box should be viewed as part of the transcriptional complex that forms when a *lea* gene is expressed in an ABA-responsive manner. Several factors determine whether the G-box element is a component of an ABRE, such as the sequence context in which the ACGT core is situated.

The bases on either side of the core sequence can affect the affinity with which different b-ZIP factors bind to the element. Additionally, since the b-ZIP proteins bind G-boxes as dimers, which may be homodimeric or heterodimeric in nature, considerable specificity may be conferred on a gene simply through the interaction of the appropriate G-box with the correct b-ZIP protein (Cuming 1999).

There are other factors that regulate ABA responsiveness of *lea* gene expression. For example, there may be *cis*-acting sequences (e.g., the RY element, CATGCATG) that combine with the G-box elements to modulate ABA-mediated expression, or there may be the additional requirement for a second transcription factor. In maize and *Arabidopsis* seeds, a second transcription factor, encoded by the *VP-1* and the *ABI3* genes, respectively, is almost universally required (McCarty et al. 1991, Giraudat et al. 1992). Examples of exceptions are the group 5 *lea*, *RAB28*, and the group 3 *lea*, *mIg3*, that are expressed in an ABA-responsive manner in embryos of *VP-1* mutants (Pla et al. 1991, Thomann et al. 1992). The means by which the VP-1/ABI-3 protein activates ABA responsiveness in seeds is not yet known. No sequence-specific DNA binding activity has been associated with it, although it can activate b-ZIP-ABRE-mediated gene expression (Cuming 1999). The transcription factor is also apparently able to activate the *cis*-acting RY element found in the promoters of the globulin *SSP* genes and some *lea* genes, which lack a G-box motif but nonetheless are ABA inducible (Cuming 1999).

6.4.2 *Developmental Gene Loci Involved in Desiccation Tolerance* (ABA-Independent Control)

Seed maturation in *Arabidopsis thaliana* is controlled by four master developmental loci: *ABI3*, *FUS3*, *LEC1*, and *LEC2*, of which the first three are

involved in desiccation tolerance (To et al. 2006). *ABI3*, *FUS3*, and *LEC2* encode related plant-specific transcription factors containing a conserved B3 binding domain, which can mediate sequence-specific DNA binding in vitro and is critical for gene activation at low or insignificant ABA concentrations. *LEC1* encodes for the CBF-A unit of the CCAAT binding trimeric transcription factor (Lotan et al. 1998). Regulation of these genes and their interactions in bringing about desiccation tolerance are still poorly understood. The summary presented in Fig. 6.2 is a synthesis of work of To et al. (2006) on various *Arabidopsis* mutants. Those authors showed that the gene regulatory controls in the network act locally, rather than on the whole embryo, and that functional redundancies exist that are also tissue specific, all of which complicates the understanding of the overall control.

However, in brief, both *LEC1* and *LEC2* control *ABI3* and *FUS3* expression in cotyledons. *FUS3* expression is controlled by a set of local and redundant regulations that vary spatially throughout the embryo and involve regulation by *ABI3*, *LEC2*, and *FUS3* itself. In the root tip, *FUS3* expression is redundantly controlled by *LEC2* and by *FUS3* itself; in the axis, *FUS3* expression is controlled by *LEC2* and *ABI3*; and in the cotyledons, *FUS3* expression is under the control of all three regulators. *ABI3* expression is regulated by *FUS3* and by itself. The downstream effects of these gene regulators are shown in yellow in Fig. 6.2. *FUS3* and *ABI3* are involved in expression of *SSP* genes, and many of those involved in desiccation tolerance in general, with *FUS3/ABI3* double mutants being desiccation sensitive. *LEC1* and *ABI3* are involved in ABA sensitivity, and ABA-sensitive mutants do not accumulate SSPs and some LEAs. *FUS3* and *ABI3* might be indirectly involved in ABA synthesis in the embryo. *LEC2* does not appear to have a direct role in the acquisition of desiccation tolerance but strongly regulates genes involved in SSP synthesis.

6.4.3 Final Comments on Control Aspects

Transcriptome profiling studies, such as the recently published work on *Medicago truncatula* (Buitink et al. 2006) and *Linum usitatissimum* (flax) (Guterierrez et al. 2006), are facilitating increasing characterization of transcription factors that might be involved in the acquisition of desiccation tolerance. For example, Guterierrez et al. (2006) have identified 13 apparently seed-specific regulators of gene expression in flax seeds that have not previously been reported. Furthermore, analysis of the proteome of such seeds is providing insight into potential translational and post-translational control of desiccation tolerance (Boudet et al. 2006). Those authors show that two LEA-like proteins (MtPM25, group 5, and MtEM6, group 1), expressed during the acquisition of desiccation tolerance in *M. truncatula*, become increasingly

structured during drying, adopting α helical and β sheet conformations that are thought to facilitate their protective functions in the dry state. Röhrig et al. (2006) have elegantly demonstrated that phosphorylation of two LEA-like proteins from the resurrection plant *C. plantagineum* (CDeT11-24, which is dehydration and ABA responsive; and CDeT6-19, a group 2 LEA) occurs upon dehydration, and this, in turn, facilitates their protective function during desiccation. In CDeT11-24, phosphorylation stabilizes coiled-coil interactions of the protein with itself (and possibly with other proteins), which in turn might facilitate the development of a protein network that could stabilize the cytomatrix in the dry state as we have postulated earlier.

6.5 Concluding Comments

The phenomenon of the acquisition and maintenance of seed desiccation tolerance is extremely complex. In this contribution, we have considered the aspects that have received focused attention in recent years, viz. the implication of proteins—particularly the LEAs and HSPs—that are accumulated, the occurrence of high sucrose concentrations, the matter of intracellular glass formation (vitrification), and the roles of ROS and antioxidants, in both signaling and intracellular damage. As briefly discussed at the outset, there are undoubtedly other mechanisms and processes that are involved in the acquisition and maintenance of desiccation tolerance—and, indeed, the maintenance of seed viability in the desiccated state. Currently, however, although the miscellany of necessary events is recognized, much remains to be elucidated for an integrated understanding of the ability of orthodox seeds to withstand severe dehydration and to survive while desiccated. Understanding of control of the various processes is beginning to emerge, but again, it will only be when a detailed and integrated picture emerges that we will really comprehend the remarkable phenomenon of desiccation tolerance of seeds and vegetative tissues.

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Fig. 3.1 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) dicotyledonous resurrection plants *Craterostigma wilmsii* (A, B) and *Myrothamnus flabellifolius* (C,D). Inset, dry leaves of *M. flabellifolius*, showing leaf curling and retention of chlorophyll in the shaded (adaxial) surfaces and waxy anthocyanin containing outer (abaxial) surfaces.



Fig. 3.2 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) monocotyledonous resurrection plants *Xerophyta viscosa* (A, B) and *Xerophyta humilis* (C,D).



Fig. 3.5 Photographs of hydrated (A,C) and dry ($\leq 5\%$ RWC [B,D]) grasses *Eragrostis nindensis* (A, B) and *Sporobolus stapfianus* (C,D).



Fig. 3.11 Desiccation pruning in *M. flabellifolius*. Desiccation causes xylem cavitation and refilling by root pressure and capillarity is effective only up to a height of 3m (Sherwin et al., 1998). Thus plants that are able to grow >3 m in the rainy season frequently cannot refill branch tips resulting in “desiccation pruning.”

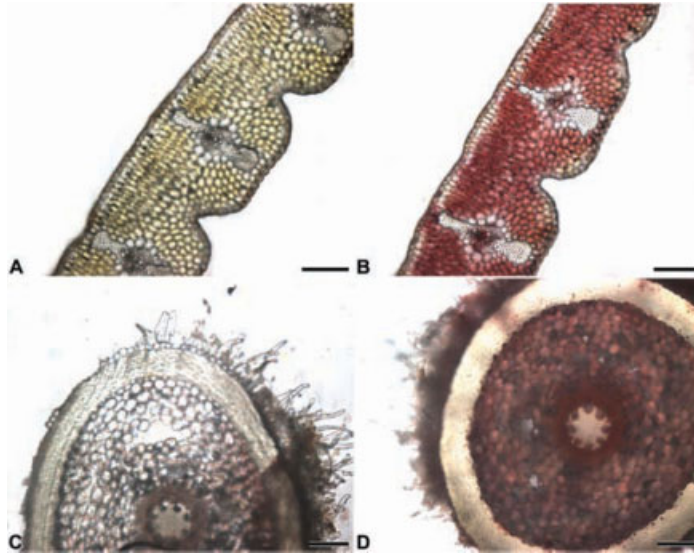


Fig. 3.14 Sucrose localization (B,D) in hand cut, unfixed, cross sections of dehydrating leaves (A,B) and roots (C,D) of *X. humilis*. Leaf RWC is 20% RWC (0.25 g.g^{-1}) and root RWC is 10% (0.12 g.g^{-1}). Sucrose visualization was using the colorimetric method of Martinelli (2007) in which the presence of sucrose (panels B,D) is identified by the red formazan precipitation from the reduction of tetrazolium (iodonitrotetrazolium chloride) upon coupling of a reaction in which sucrose is the substrate. Control sections (A,C) were not exposed to INT. Sucrose is mainly cytoplasmic. Scale bar for all images = $200 \mu\text{m}$.

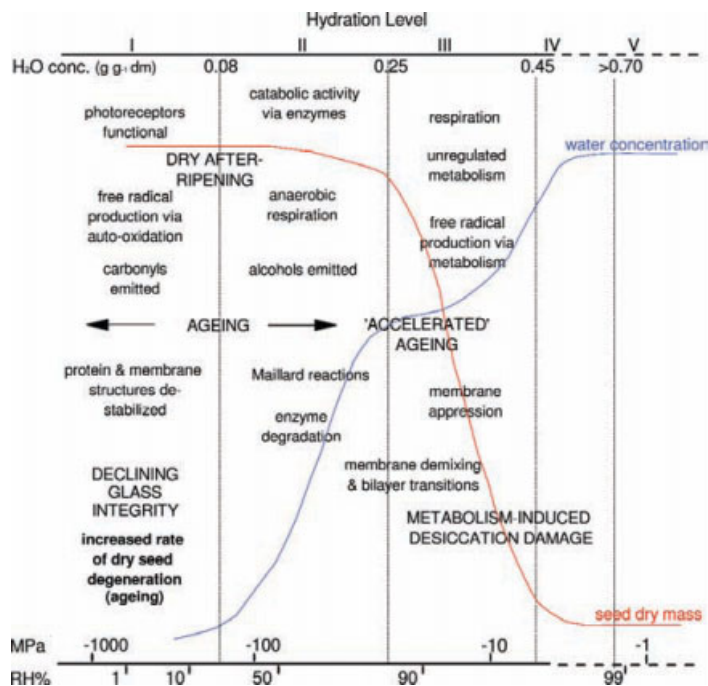


Fig. 6.1 The trends of dry mass accumulation (to a plateau) and decline in water concentration during seed maturation are depicted in relation to the events that do, or can, occur through the hydration levels III to I. Approximate water concentrations ($\text{g} \cdot \text{g}^{-1}$ dry mass) at the boundaries of the hydration levels appear at the top of the Fig., with equivalent water potentials (Ψ) and equilibrium RH values (%), below. (Modified after Vertucci and Farrant 1995, Walters et al. 2005, Berjak 2006).

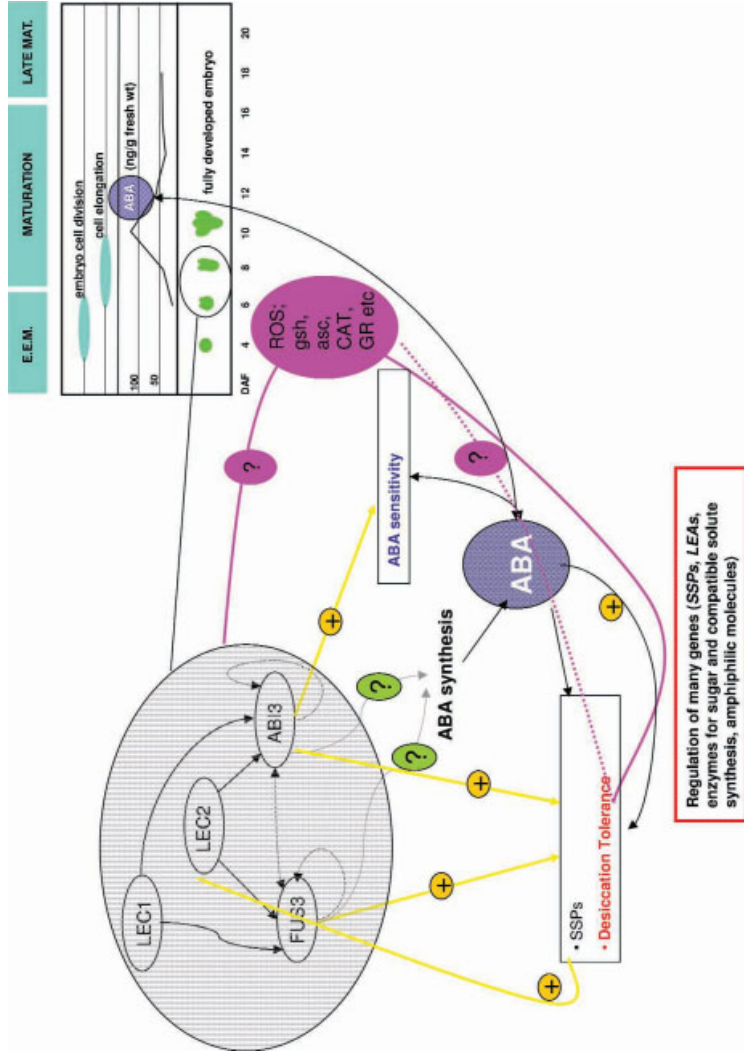


Fig. 6.2 Stages of development (early embryo morphogenesis, EEM, and maturation, MAT) and ABA accumulation in seeds of shown at the *top right* (adapted from Vicente-Carbajosa and Carbonero 2005). A summary of the master gene control of maturation e acquisition of desiccation tolerance, within the embryo (from the heart-stage onward) are given in the *gray oval* on the left. Control netw ter genes are depicted by *black lines*; *solid lines* depict control of one locus over another; and *dashed lines* depict regulatory influences among them and upon themselves. Downstream actions of the genes are given as *yellow lines*. Control via ABA and ROS are shown in *blue* and pathways are represented by question marks. LEC = Leafy cotyledon; FUS3 also belongs to the leafy cotyledon phenotype, chomes; ABI3, abscisic acid insensitive; gsh, glutathione; asc, ascorbate; CAT, catalase; GR, glutathione reductase; SSPs,

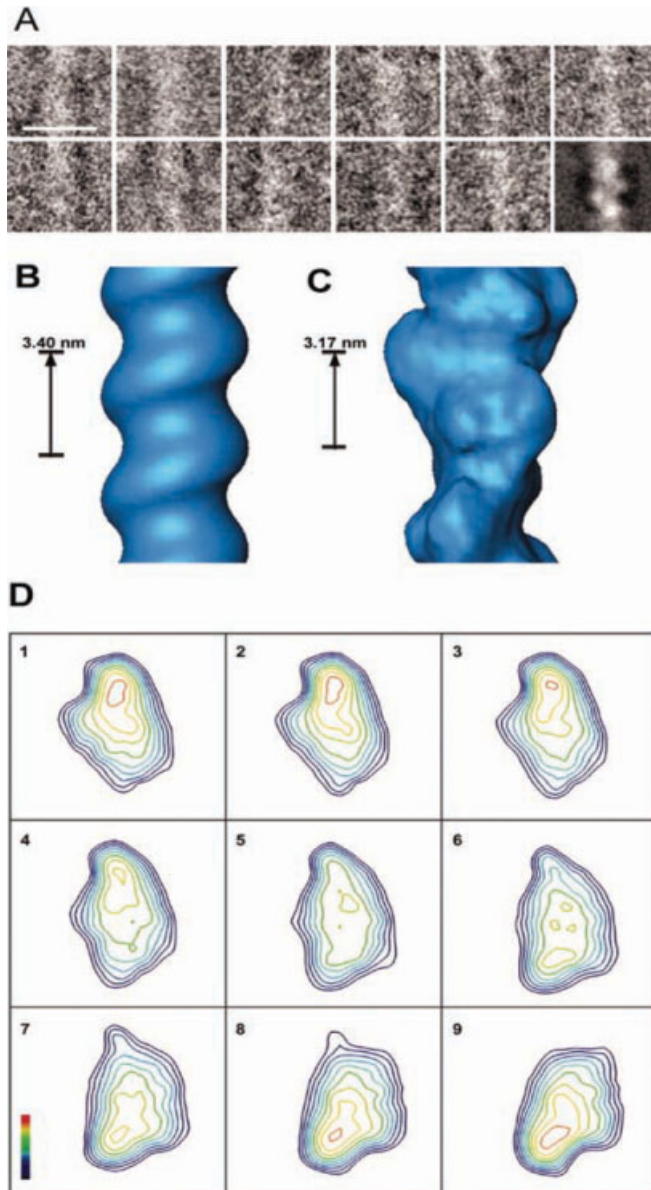


Fig. 8.2 DNA-SspC 3D reconstruction. (A) Gallery of raw "particles" (helix sections) that have been aligned by rotation and translation. The last frame shows the average of all aligned particles belonging to one representative class from the reconstruction. (B, C) Surface representation of the initial model (B) and final reconstruction (C). (D) Slices through the reconstruction (perpendicular to the helical axes). Consecutive slices are separated by 0.235 nm and cover one helical subunit. After Frenken-Krispin et al, 2004.

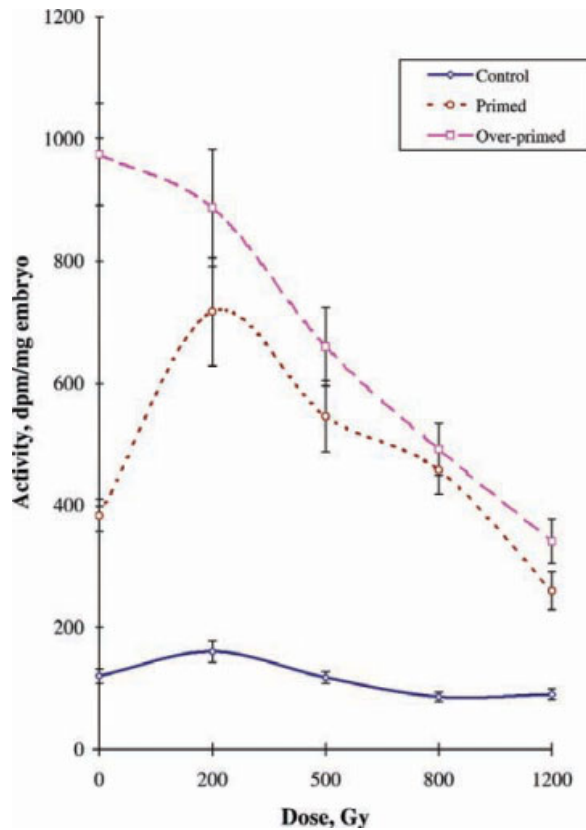


Fig. 8.5 Unscheduled DNA synthesis (measured by incorporation of H^3 -thymidine) in differently primed sugar-beet seeds after gamma-irradiation. Control, unprimed seed; primed, seed hydrated to 25% moisture content; overprimed, seed hydrated to 33% moisture content.

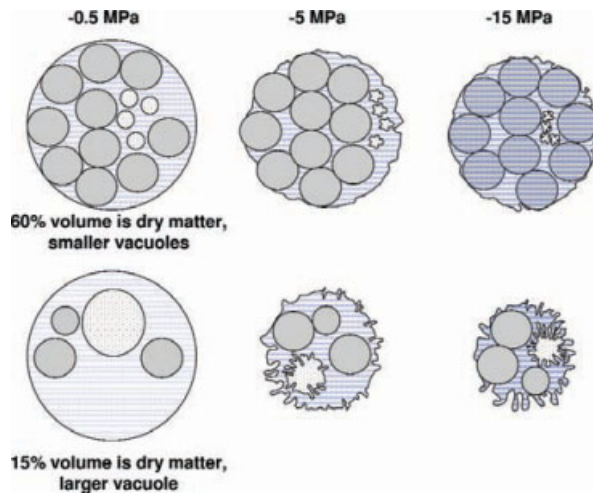


Fig. 9.4 Scaled drawing of osmotic shrinkage in cells containing different quantities of dry matter reserves. Diagrams are representative of the mature cotyledon and immature embryo described in Fig. 9.2. Cell volume was calculated assuming a 1:1 loss of mass to volume as water is removed. Changes in vacuole (light-colored circles with dots) volume were calculated assuming that 10% of the original volume was comprised of solutes.

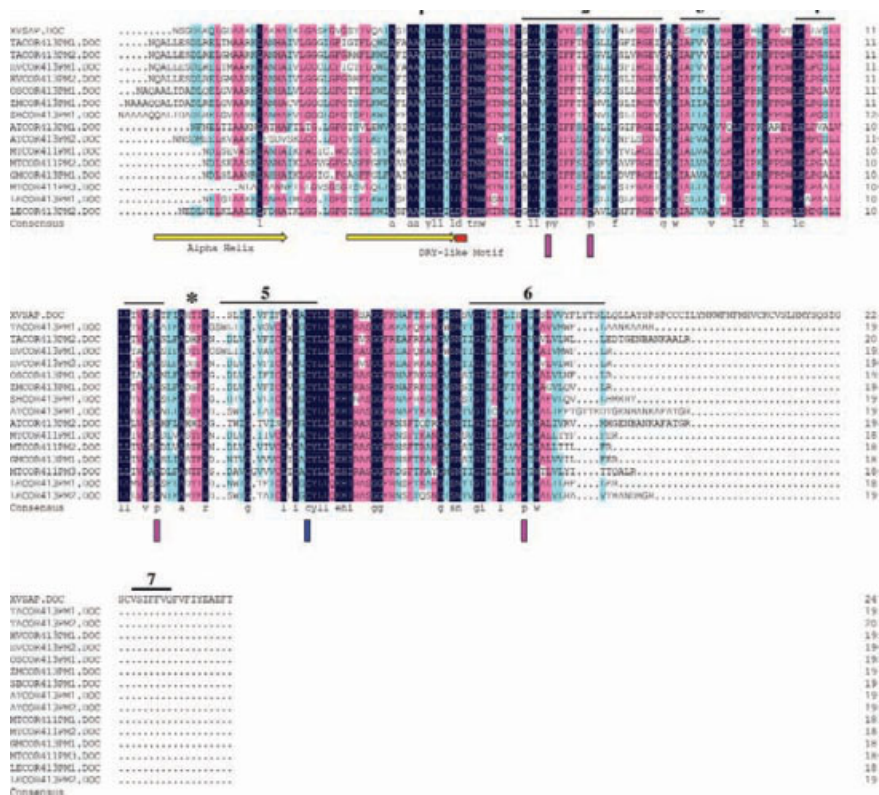


Fig. 10.1 Multiple sequence alignment of the *XvSap1* protein sequence with COR413-PM proteins. The proteins are *TaCOR413PM1*, *TaCOR413PM2*, *HvCOR413PM1*, *HvCOR413PM2*, *OsCOR413PM1*, *ZmCOR413PM1*, *SbCOR413PM1*, *AtCOR413PM1*, *AtCOR413PM2*, *MtCOR413PM1*, *MtCOR413PM2*, *GmCOR413PM1*, *MtCOR413PM3*, *LeCOR413PM1*, and *LeCOR413PM2*. The respective GenBank/EST accession numbers are AAB18207, AAL23724 (BE421687 and AL502741) (BG343566 and BF628071), AF283006, AY181208 (BI075784 and AW680600), AF283004, AF283005 (BF003463) (BG647116) (BE211677 and AW309837) (BG456396 and AL384664) (AW039062) (BG642925, AW041686, and BG629922). The putative *XvSap1* TMDs are indicated by a line above the amino acid sequence and labelled using the DAS-TM prediction result (see Fig. 10.2). The *XvSap1* and COR413 proteins phosphorylation site is indicated by an asterisk.

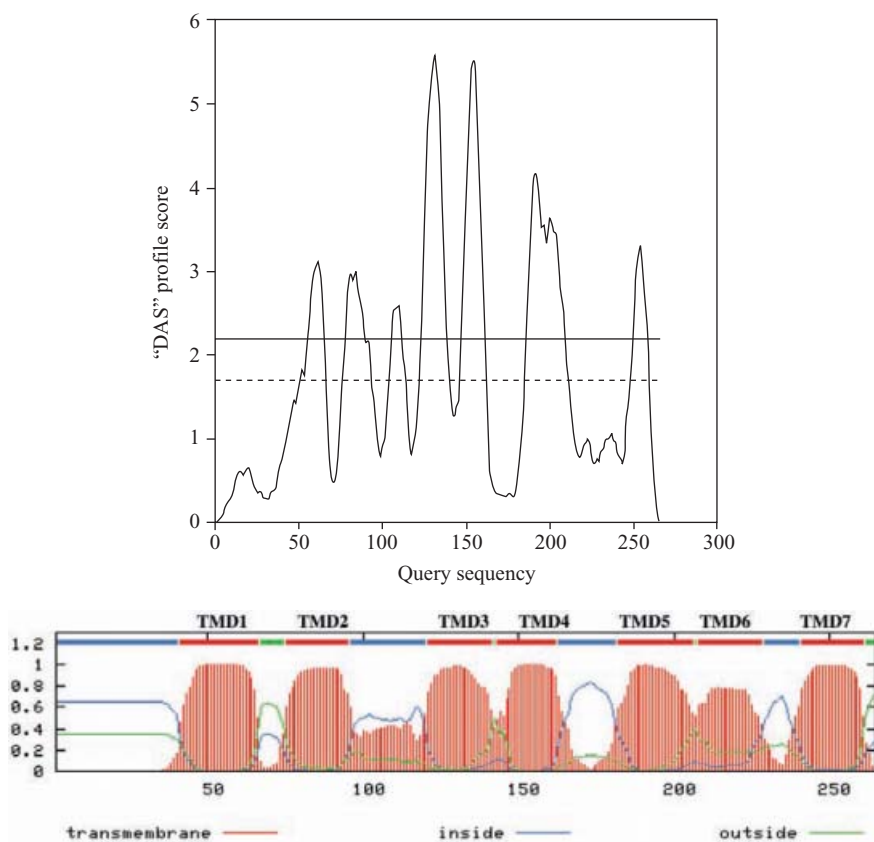


Fig. 10.2 Hydropathy and transmembrane predictions. (A) DAS-TM prediction. The *dashed line* represents the loose cutoff; *solid line* represents the strict cutoff. (B) TMHMM topology profile. The transmembrane helices are indicated above the profile.

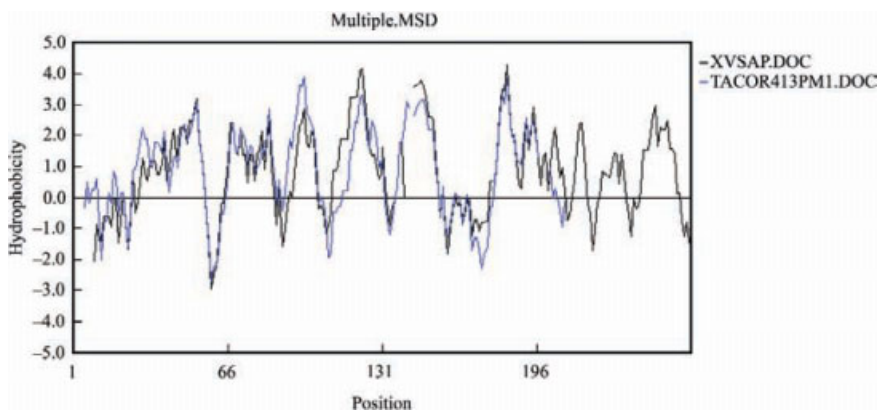


Fig. 10.3 Hydrophobicity overlap profile of *XvSap1* with TaCOR413PM protein as determined by the method of Kyte and Doolittle (1982).

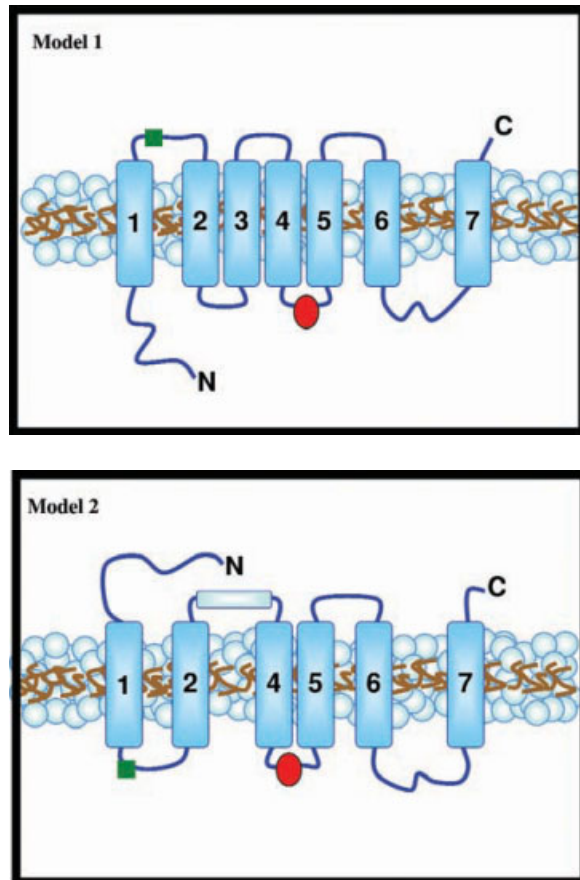


Fig. 10.5 Proposed models for the *XvSap1* protein. *Blue boxes and blue lines* indicate TMD and interconnecting loops, respectively. *Red circle*, phosphorylation site. *Green box*, position of the highly conserved DRY triplet motif.

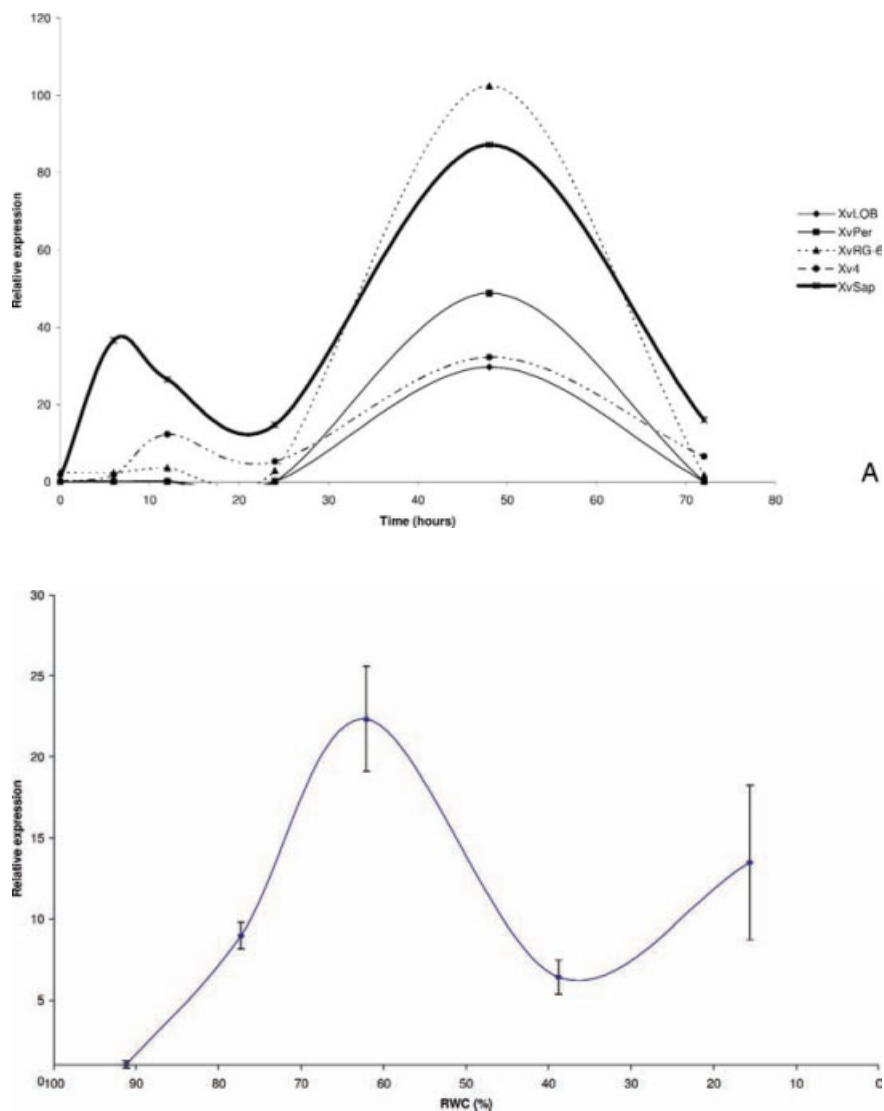


Fig. 10.6 Expression profile curves of *XvSap1* transcripts using qPCR analysis. **(A)** Expression profile following exogenous treatment of an *X. viscosa* plant with ABA. The *XvSap1* profile is displayed in **bold**. **(B)** Expression profile following a dehydration treatment of an *X. viscosa* plant.

7 The Glassy State in Dry Seeds and Pollen

Olivier Leprince and Julia Buitink

7.1 Introduction

The corollary of desiccation tolerance is the ability for anhydrobiotes to survive a certain period of time in the dry state. The notion of longevity in the definition of desiccation tolerance is rather vague. From an ecological point of view, longevity of dry anhydrobiotes is determined by the necessary time to bridge unfavorable conditions (Farrant and Kruger 2001, Hoekstra 2005, Walters et al. 2005a). From the point of view of germplasm bank curators, the ability to survive drying is only practically meaningful if the dry organisms are able to remain alive for the time equivalent of a human generation. In fact, anhydrobiotic organisms differ considerably with respect to their life spans in the dry state: they typically remain viable for years to centuries (Kivilaan and Bandurski 1981, Priestley et al. 1985, Hoekstra 2005, Walters et al. 2005a, 2005b). The most remarkable discovery involved ancient seeds of sacred lotus from China; radiocarbon dating showed these seeds were 1288 ± 7 years old while still capable of germinating (Shen-Miller et al. 1995). The foremost explanation for high stability of dry anhydrobiotes is their ability to withstand the removal of water. Whereas the mechanisms that enable anhydrobiotes to survive the removal of their cellular water are discussed elsewhere, this chapter attempts to explore how these organisms can survive the dry state for long periods of time.

Over the past years, two hypotheses attempted to understand the kinetics of aging during storage of various dry systems (food stuff, pharmaceuticals) and the influence of temperature and water thereof: the “water substitute hypothesis” and “the glass hypothesis” (Walters 1998). These hypotheses are not mutually exclusive (see, e.g., Buitink et al. 1998b, Chang et al. 2005, He et al. 2006). The water substitute hypothesis states that protective molecules such as sugars can form hydrogen bonds on the surface of macromolecules and substitute for the thermodynamic stabilization function of water that is lost during drying. Aging reactions in dry seeds and pollens are thus controlled by the thermodynamic status of the residual water (reviewed by Walters 1998). While water-binding models help to understand the relations between storability, water content, and temperature, they do not directly address how the kinetics of the chemical reactions explains the loss of viability (Walters 1998). The “glass hypothesis” relies on the principle that macromolecules are

immobilized to a certain extent in a rigid matrix, which limits molecular mobility that drives deteriorative reactions in the dry state. By analogy with studies on dry food systems, the rigid matrix in dry anhydrobiotes should correspond to a glassy state formed by the cytoplasmic solutes during drying (Burke 1986). Their high viscosity, a canonical property of glasses (Angell 1995), may stop all chemical reactions that require molecular diffusion (Burke 1986, Walters 1998, Buitink and Leprince 2004). The glass hypothesis further states that in dry anhydrous organisms, glasses are formed from cell solutes like sugars that are known to provide protection from denaturation of large molecules and formation of molecular aggregates (reviewed in Buitink and Leprince 2004). The same conditions that are known to increase longevity (i.e., low water content and temperature) are also beneficial to the formation of a glassy state. Hence, over the past two decades, many attempts have been made to explain aging kinetics through the presence and specific properties of intracellular glasses.

By establishing analogies with the material and food science, we describe how to detect glasses and show that the glass-to-liquid transition is not a parameter that holds the key to our understanding of aging kinetics during storage. We argue that other parameters linked to the properties of the glassy state are more likely to be relevant than the glass transition per se. Unraveling these properties has brought new insights into the storage behavior of dry anhydrobiotes. For example, we document that mobility in the glassy state is reduced but not stopped. Because glass properties are the result of its composition, we discuss how cytoplasmic solutes contribute to the glass formation.

7.2 Definition, Formation, and Detection of Glasses

A *glass* corresponds to a physical solid but a thermodynamic liquid. *Solids* can exist in a crystalline or amorphous form. *Crystalline materials* have defined structures, stoichiometric compositions, and melting points. By contrast, *amorphous materials* have no defined structure. The three-dimensional long-range order that normally exists in a crystalline material does not exist in the glassy state, and the position of molecules relative to one another is more random, comparable with that in the liquid state (Angell 1995, 1998). Glass formation can be considered as an intrinsic property of all liquids, including water and aqueous solutions, given only that the formation of the crystalline phase is prevented. A glass is usually referred to as a supercooled liquid with an extremely high viscosity—typically in the order of 10^{14} Pa • sec (Franks et al. 1991, Angell 1995). Due to the high viscosity of the glassy matrix, the return to equilibrium occurs at such a slow rate that a glass is also considered as “semi”-equilibrium state. This phenomenon is referred to as *relaxation* or

physical aging. Examples of well-known and thoroughly studied molecules that can undergo glass transitions can be found in the food, polymer, and material sciences and include sugars, proteins, starch, complex food systems, and a wide range of polymers (Sperling 1986, Levine and Slade 1991, Roos 1995, Lourdin et al. 1997, Chanvrier et al. 2005, Ubbink and Krüger 2006).

As we know from the physical science, glasses can be formed by many different routes (Angell 1995), and their function is also referred to as vitrification (Fig. 7.1B, C). In plant anhydrobiotes, the transformation of the cytoplasm into a highly viscous, glassy state is naturally occurring during air drying that takes place during the later stages of development. For example, the removal of water from pea seeds below 30% (dry weight basis) induces a rapid and steep rise in viscosity of the cytoplasm by several orders of magnitude, typical of a glass forming system (Fig. 7.1B). At room temperature, the cytoplasm of anhydrobiotes turns into a glassy state when the tissues are equilibrated below approximately 65% relative humidity (RH) (Leprince and Walters-Vertucci 1995, Buitink and Leprince 2004, Lehner et al. 2006). Rapid cooling by plunging wet tissues into liquid nitrogen is an alternative route to glass formation and is used for cryopreservation of both desiccation-tolerant and sensitive organisms (Walters et al. 2005b). For both routes, glass formation can be explained by the rapid increase in viscosity, which is thought to induce a supersaturation of the cytosolic components, leading to an increase in the cohesive forces between molecules, restriction of the molecular mobility within the cytoplasm, and, in the case of cooling, inhibition of nucleation and crystallization. At some point, the liquid passes through the “glass transition,” which is generally considered to be a range of temperatures over which the system falls out of equilibrium and is stuck. The range corresponds to that needed to change the average relaxation time by two or three orders of magnitude, usually between 100 and 0.1 second (Angell 1995, 1998). Thus, assessing the temperature at which a substance transforms from a liquid into a glass or vice versa (the glass transition temperature [T_g]) has been the most popular way to characterize biological glasses. It is noteworthy that the definition of T_g is arbitrary and is not ruled by an international convention (Angell 1995).

Various techniques exist to detect T_g , all based on the sudden change in the physical properties of the liquid during cooling or heating, usually at an arbitrary rate of 10°C/min (reviewed in Williams 1994, Buitink and Leprince 2004, Leprince and Golovina 2002). The most common technique used to measure T_g is differential scanning calorimetry (DSC), which detects the glass transition by a discontinuity in specific heat during constant heating of a glassy material (Franks et al. 1991). Other techniques that have been applied to detect glasses in dry biological tissues are thermally stimulated depolarization current method (Bruni and Leopold 1992, Konsta et al. 1996), electron paramagnetic resonance (EPR) spectroscopy (Buitink et al. 1998a, 1999), and

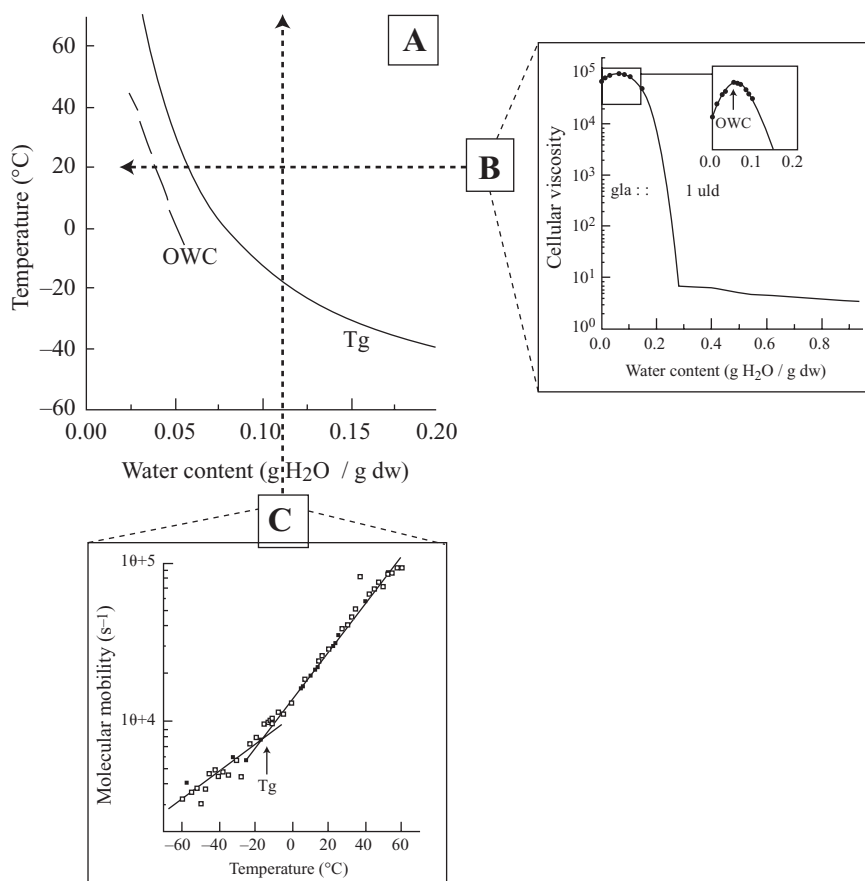


Fig. 7.1 Three angles on intracellular glass in pea embryonic axes. (A) State-diagram depicting the effect of plasticization of water on the glass transition temperature (T_g , solid line) determined by differential scanning calorimetry during heating at 10°C together with the temperature/water combinations resulting in optimum storage conditions. Prior to measurement, pea seeds were equilibrated at different relative humidities (data derived from Buitink et al. 1999). (B) Increase in cellular viscosity (fold increase) during drying while the temperature is held constant at 20°C. Viscosity values were derived from EPR spectroscopy measurements of the rotational motion of a hydrophilic spin probe that was loaded into the cytoplasm (data derived from Buitink and Leprince 2004). The inset shows the 0 to 0.2 g water • g dry weight⁻¹ region where the cellular viscosity reaches a maximum, which corresponds to the optimum water content for storage at 20°C. (C) Effects of heating on the molecular mobility for axes containing 0.12 g water • g dry weight⁻¹. The molecular mobility was assessed by measuring the rotational correlation time of a spin probe embedded in the glassy matrix at a heating rate of 10°C/min. The arrow indicates the onset of the T_g as determined by the break between in the linear temperature dependence of the rotation of the spin probe (data derived from Buitink et al. 1999). OWC, optimum water content and temperature for storage.

dynamic mechanical thermal analysis (Maki et al. 1994). The advantages and limitation of these techniques have been discussed elsewhere (Leprince and Golovina 2002, Sun 2002). Among techniques that experimentally provide further insights into the molecular properties of glasses, EPR and Fourier transform infrared (FTIR) spectroscopy have been successfully applied to biological glasses. EPR spectroscopy gives access to the rotational correlation time (t_R) of guest molecules (so-called spin probes) that are inserted in the cells. From the t_R values, a measure of local viscosity can be derived (Buitink et al. 1999, Van den Dries et al. 2000). A short t_R implies a high molecular (rotational) mobility. An application of this technique is saturation transfer EPR, which provides access to very slow rotational mobility, corresponding to that in the glassy state. Using FTIR spectroscopy, one can assess the extent of molecular interactions through hydrogen bonding and thus the molecular packing of the glassy matrix components (Wolkers et al. 1998a, 1998b, Hoekstra 2005). The wave number position of OH-stretching vibrations (ν_{OH}) reveals information about the strength and length of hydrogen bonding within the glass (references in Hoekstra 2005). However, this technique can only be applied in the dry state in the total absence of water. Furthermore, caution should be exerted when FTIR is applied to seeds and pollen because the origin of the molecular contribution to the OH-stretching vibration signal is unknown and could well be originating from storage proteins (Hoekstra 2005).

7.3 Properties of Intracellular Glasses: Water, Hydrogen Bonding Network, Molecular Density, and Relaxation

Most of the properties of biologicals described in the literature have been inferred from the comparison with those of model glasses made of known composition. Many factors contribute to the complexity of intracellular glasses, which hinder the characterization of physicochemical properties at the molecular level and make it difficult to extrapolate data obtained from simple binary or ternary systems (water/carbohydrate/proteins). In addition, cellular organelles differ in the composition of their matrices. One can imagine that the glass components and properties will not be uniform within the cell or even in different tissues. For example, the physicochemical properties of the glassy mitochondrial matrix in which organic acids and proteins are preponderant would be different from that of the cytosol. Thus, different glassy matrices may exist in the cell, as evidenced by the observation that in maize, both zein, a storage protein, and starch form a glass with a respective T_g of 85° and 107°C at 33% RH (Chanvrier et al. 2005). Furthermore, the intracellular glasses are constrained by interfaces such as membrane lipids and/or proteins. Water molecules that interact at these interfaces may have different glassy

characteristics than the bulk amorphous matrix (Bryant et al. 2001, Hoekstra 2005, Weik et al. 2005). At low water contents, the contribution of interfacial interactions to the glass-to-liquid behavior would become increasingly important. In addition, the desiccation kinetics leading to the glass formation at the later stages of maturation is most likely complex. Using a microfluidic device, Aksan and colleagues (2006) were able to induce heterogeneous drying kinetics of various sugar solutions. They found that the glassy matrices exhibited spatial variations in water and solute contents that were attributed to the fact that during rapid drying, a glassy skin was formed rapidly around the liquid, thereby reducing the diffusivity of water at the core of the mixture. Whether the history of drying leading to glass formation in seed tissues leads to heterogeneous amorphous matrices is unknown but deserves further investigation.

In biological glasses, water is considered as a plasticizer and has a profound effect on T_g . The plasticizing effect of water on the T_g is best illustrated by a state diagram (Fig. 7.1A), which shows the water content/temperature combinations at which T_g is detected. Below the T_g , the mixture is in a glassy state, whereas increasing the temperature decreases its viscosity and results in melting of the glass (Fig. 7.1C). A plasticizer can be defined as a material incorporated in a polymer to increase the polymer's workability, flexibility, or extensibility (Sears and Darby 1982). Characteristically, the T_g of an undiluted polymer is much higher than that of a low-molecular-weight glass-forming diluent. As the diluent concentration of the solution increases, T_g decreases (Fig. 7.1A), because the average molecular weight of the homogeneous polymer-plasticizer mixture decreases, and its free volume increases (Ferry 1980, Kilburn et al. 2004). Plasticization, on a molecular level, increases intermolecular space, decreases local viscosity, and increases mobility. A slight increase in water content of various sugar glasses from approximately 3% (dry weight basis) to 7% resulted in a large increase in mobility at T_g (Buitink et al. 1999). Recently, Kilburn et al. (2004) studied the molecular mechanism of plasticization of sugar glasses using positron annihilation lifetime spectroscopy. According to these authors, the plasticization proceeds via a complex mechanism involving hydrogen bonding formation and disruption close to the carbohydrate chains and changes in the matrix free volume (Kilburn et al. 2004). Apart from the plasticizing effects of water, the properties of water molecules trapped in the glassy state vary with the residual amount and temperature. In dry anhydrobiotes, this can be derived from water sorption isotherms and measurements of heat capacity (Buitink et al. 1996, 1998b, Walters 1998). In pollen, the relation between C_p of water and temperature was found to be parallel to the T_g curve. Furthermore, the C_p curve overlaps the Brunauer-Emmett-Teller (BET) monolayer, which corresponds to the water content at which the strong binding sites are saturated (see Walters 2004 for a discussion on its applicability to biological material). According to Kilburn et al. (2004), the

sorption behavior of water molecules in amorphous matrices made of maltodextrin (a polymer sugar) is due to the formation and disruption of hydrogen bonds. The water in the glassy matrix is apparently absorbed via a localized binding to small moieties close to the carbohydrate chains. This indicates that the “glass hypothesis” and the “water substitute hypothesis” are linked. Furthermore, the absorbed water appears to fill the voids within the glassy matrix, resulting in an increased density (Kilburn et al. 2004).

The composition of dry glasses also influences its density (see later). Hydrogen bonding and molecular packing of the glassy matrix components of various anhydrobiotes have been compared with that of glasses made of monosaccharides, disaccharides, and trisaccharides (Wolkers et al. 1998a, 1998b, Buitink et al. 1999, Hoekstra 2005). Comparison of the molecular mobility at T_g with the hydrogen bonding network at T_g revealed that in dry glasses made of low-molecular-weight sugars such as sucrose, the resulting dense hydrogen bonding network was accompanied by a slow rotational motion (Buitink et al. 1999, 2000a, 2000c, Van den Dries et al. 2000). Conversely, in oligosaccharide glasses (e.g., stachyose), characterized by a less-dense hydrogen bonding network, the rotational motion was faster. It is known that the molecular free volume at T_g increases with increasing molecular weight for small polymers/oligomers (Fox and Loshaek 1955, He et al. 2006). Also, the average radius of molecular free volume holes at T_g increases with T_g (Bartos 1996, Li et al. 1999). Thus, it is suggested that sugars of high molecular weight will form a glass matrix with a higher molecular free volume, in which small molecules and water are less hindered in their motion compared to a glass matrix formed by the disaccharide sucrose. This suggestion is supported by He et al. (2006), who demonstrated that the free volume model could predict viscosity and diffusion in sugar glasses and found divergences of storage stability between monosaccharides and disaccharides. Interestingly, when local, fast dynamics are suppressed by low- T_g diluents, the glassy mixture shows enhanced protein stabilization (Cicerone and Soles 2004). In seed tissues, the hydrogen bonding network appears to be much denser than in sugar glasses and is now thought to be a reflection of the protein content (Hoekstra 2005).

In polymer glasses, it is long known that the glassy relaxations also depend on how the glass was reached (Angell 1998). Molecular dynamics simulations reveal that viscous liquids can reach many different metastable states upon cooling. The number of these states depends on how the collection of N particles are arranged when the system is stuck in the glassy state (Angell 1998). There is also the possibility for hopping from one metastable state to another, providing that the temperature of the system is increased to allow the system to slowly explore them, hence restoring an equilibrium state (Angell 1998, Marqués and Stanley 2005). This is called relaxation, annealing, or aging, depending on whether one works with liquids, glasses, or polymers, respectively. Thus glasses can

experience different relaxation rates—in other words, exhibit different mobility characteristics—according to the particular metastable state in which the glass is stuck. There is no such theoretical concept that is translated yet for intracellular glasses. Given the complexity of the cellular composition, we do not know whether it is even feasible. However, we can easily imagine that many metastable states can be formed locally within a cell depending on the conditions of the drying rates and local molecular composition. Therefore, it would be interesting to determine whether different drying rates of seeds, for instance, during maturation drying on the plant, would have an impact on the glassy properties and corresponding aging rates. This is warranted by the recent observation that the degree to which cryogenic temperatures prolong life span of lettuce seeds depends on how the seeds were handled before storage (Walters et al. 2004). Also, using DSC, we earlier found that the heat capacity associated with T_g of glasses in bean embryonic axes was different according to whether the glassy matrix had melted prior to the analysis (Leprince and Walters-Vertucci 1995).

7.4 Glasses Enhance Structural Stability and Life Span of Dry Anhydrobiotes

Many studies on model systems have shown that glasses retain the activity of enzymes and conformation of proteins (reviewed in Blanshard and Lillford 1993, Roos 1995, Hancock and Zografi 1997, Crowe et al. 1998, Sun and Davidson 1998). In the pharmaceutical and food industry, it is recognized that the presence of an amorphous state has very important implications for storage (Hancock and Zografi 1997, Ubbink and Krüger 2006). For example, the suppression of local motions correlates precisely with the effectiveness of the glass at stabilizing labile enzymes (Cicerone and Soles 2004). By measuring both the deactivation dynamics of alcohol dehydrogenase and molecular motion in the MHz-GHz range in the glassy state, these authors were able to show that there is a coupling between the dynamics of the glass host and stabilization of the proteins. What is remarkable in these observations is that there is a separation of 16 orders in magnitude in time between the enzyme lifetime embedded in the sugar glass (10^7 seconds = 3 months) and the local motion of the glassy matrix (10^{-9} seconds). Similar results on lysozyme embedded in glucose-water glass have been reported (Marconi et al. 2005), suggesting that macromolecules are “slaved” to their environment when they are in a glassy matrix. In dry biological tissues, there is also direct evidence that a glassy state retains structural and enzymatic integrity and, as such, plays a role in long-term storage stability. Golovina et al. (1997) found that the secondary structure of proteins in dry seeds of several species of seeds appears to be very stable and remains conserved after several decades of open storage.

No protein aggregation and denaturation were found after 28 years, despite loss in viability. In somatic embryos of carrot, rapid drying leads to low survival in contrast to slow drying (Wolkers et al. 1999). However, the overall protein secondary structure of slowly and rapidly dried embryos resembles one another to a large extent. This absence of protein denaturation can mainly be explained by the formation of a glass in both drying regimes, which prevented changes in protein conformation. Glasses also participate to the stabilization and protection of phospholipid model systems. Formation of an amorphous sugar matrix between membranes limits their close approach and offers a mechanical resistance against the desiccation-induced lipid phase transition (reviewed in Bryant et al. 2001). Glasses inhibit membrane fusion, whereas the depression of the melting transition temperatures of the membrane lipids can additionally be caused by direct hydrogen bonding of the sugar with the phospholipid head groups (Sun et al. 1996, Crowe et al. 1997, 1998, Bryan et al. 2001, Cacela and Hinch 2006).

It is important to realize that the water content at which glass formation occurs during drying at room temperature in developing seeds is approximately 10% to 15% moisture (Buitink and Leprince 2004, Lehner et al. 2006) (Fig. 7.1B). A number of species exist with seeds and pollen that cannot survive the removal of protoplasmic water. In both these desiccation-sensitive seeds and pollen, the cytoplasm transforms into glass in the dry state despite the loss of viability (Sun et al. 1994, Buitink et al. 1996). In desiccation-sensitive red oak seeds, the relationship between T_g and water content was found to be indistinguishable from that of mature desiccation-tolerant soybean axes (Sun et al. 1994). Therefore, glasses do not confer desiccation tolerance per se. Apparently, dehydration-induced damage in desiccation-sensitive seeds and pollens occurs at water contents far above that at which protection of the glassy state can be effective. Apart from desiccation-sensitive seeds, some species (e.g., coffee, *Cuphea* spp.) produce seeds that can withstand drying to an extent that leads to the formation of a glassy state but for which further drying leads to loss of viability. Furthermore, these so-called intermediate seeds do not store under cold and dry conditions while in the glassy state (Crane et al. 2003, 2006, Dussert et al. 2006). Typically, at room temperature, they rapidly die when dried at RH of 30% or below (10% moisture and below), whereas at 5°C, the critical RH for survival is 50% RH. In both temperature/RH combinations, the seeds are in a glassy state (based on the data provided in Buitink and Leprince 2004). These observations suggest that the glass formation per se cannot fully explain the incurred stabilization exhibited by dry anhydrobiotes and additional factors must be taken into account. How these factors are linked to the physicochemical properties of glasses and/or seed composition are discussed later in this chapter.

Whereas cellular glass formation cannot be considered as a mechanism to confer desiccation tolerance, it plays an important role in conferring stability

during storage. Several approaches have been undertaken to assert a link between glass properties and aging rates. Typically, seed and pollen deterioration is accelerated when the tissues are not in the glassy state (Sun and Leopold 1993, 1995, Buitink et al. 1998b, Murthy et al. 2003, Walters et al. 2005a). Conditions that bring the anhydrobiotes above their T_g result in an increase in the activation energy of the aging rate within the same order of magnitude as that of rotational mobility in the cytoplasm: about a factor of 2 to 3 (Buitink et al. 1999, Walters 2004). A comparison of the molecular mobility in intracellular glasses under different storage conditions with the respective aging rate revealed a linear relationship between both parameters over a wide range of temperatures and water contents (Buitink et al. 2000b, Walters 2004). In addition, by combining theoretical considerations of thermodynamics parameters (configurational entropy) of the supercooled liquid and the glassy state with measurements of heat capacity of water in dry seeds and pollen, Walters (2004) showed a linear correlation between aging rates and structural mobility over a wide range of temperature. This relationship differs only from that observed using direct measurement of molecular mobility by the time scale (Buitink et al. 1999, 2000b, 2000c), suggesting that different types of molecular motion occurring at rates can be used to explain the rate of deterioration during storage. Finally, Walters and colleagues showed that the aging rates of more than 200 seed species during storage could be fit by the so-called Avrami equation, a model that describes cooperative reactions based on viscosity properties (Walters 1998, 2004, Walters et al. 2004, 2005a, 2005b). All these findings suggest that aging rates and thus life span of germplasm can be influenced by the molecular stability and the viscosity of the cytoplasm, signifying an important function of intracellular glasses in conferring stability during storage.

Apart from mobility, an attempt has been made to also correlate life span with the hydrogen bonding network of dry seeds. Using a range of *Arabidopsis* mutants exhibiting developmental defects, Wolkers et al. (1998c) reported a negative correlation between the density of the hydrogen bonding and longevity, suggesting a causal relationship between survival and hydrogen bonding strength. However, Hoekstra (2005) did not confirm this relationship using a wider range of plant anhydrobiotes and attributed the differences in hydrogen bonding interactions to variations in the protein content in the glassy cytoplasm.

The above observations show that cytoplasmic viscosity and molecular motion below T_g play a central role in governing the deterioration kinetics of seeds and pollen. Actually, the mobility still remains relatively low in seeds and pollen far above T_g , although the slope of the mobility-versus-temperature plot does increase by a factor of 2 to 3, indicating an increased mobility above T_g (Buitink et al. 1999, 2000b) (Fig. 7.1C). Like for sugar glasses, the determination of the temperature dependence of the molecular mobility revealed

the occurrence of a second kinetic change in mobility at a definite temperature above T_g , referred to as the critical temperature (T_c) (Buitink et al. 2000c, van den Dries et al. 2000, Buitink and Leprince 2004). The occurrence of T_c has been associated with the “collapse temperature” of glasses, a phenomenon that is due to a strong reduction in viscosity such that a flow on a practical time scale is observed (Roos 1995, Sokolov 1996, Sun 1997). One of the consequences of the existence of T_c is that models that describe viscosity or molecular mobility above T_g are valid only above T_c (Rössler et al. 1988). While T_c appears to be ubiquitous in glasses (Sokolov 1997), it was not detected in biological glasses within the temperature range of 0° to 50°C above T_g using EPR spectroscopy (Buitink et al. 2000c). In accordance with this, proteins in dry intracellular glasses of seeds and pollens of several species were not found to denature up to a temperature of 60°C above T_g (Hoekstra 2005). When the rate of vigor loss or the rate of degradative reactions of mungbean seeds was plotted as a function of the difference between the experimental temperature of storage and T_g , a sudden break was found in this relation at approximately 40°C above T_g (Murthy et al. 2003). This break was interpreted by the authors as the collapse temperature. From the above observations, two important issues arise. In the absence of T_c within the range of temperature that are of biological significance, the application of the Williams-Landel-Ferry (WLF) equation in dry anhydrobiotes should be considered with great caution. The WLF equation is usually applied when the temperature dependence of viscosity or mobility above T_g cannot be described by an Arrhenius-like relationship (Sun 1997, Buitink et al. 1999, Walters 2004). The second issue pertains to the ecophysiological implications of a high T_c for the long-term survival of seeds and pollen (as well as resurrection plants) in their natural environment. Under ambient conditions (20° to 25°C, 50% to 60% RH), seed tissues are around their T_g . Any fluctuations resulting in an increase in RH or temperature will bring the tissues above T_g but will not have a great impact on the cytoplasmic viscosity, thereby decreasing the chances for collapse, crystallization, and loss of macromolecule integrity during excursions above T_g . This might explain the long life span of dry biological tissues recorded under laboratory conditions (Walters et al. 2005b).

Another characteristic that shapes the properties of glasses is the concept of “fragile” and “strong” glasses, which are derived from the behavior of the properties of a viscous liquid submitted to rapid cooling (see Angell 1998, Sastry 2001, Marqués and Stanley 2005 for references on the physical basis of fragility). Fragile glasses experience a sharp change in liquid properties (such as viscosity) in a narrow range of temperatures. This is reflected by, for example, a jump of heat capacity at T_g , a rapid increase in molecular mobility during drying, and by the presence of a collapse temperature (Angell 1998, Sastry 2001, Marqués and Stanley 2005). In contrast, strong glasses have a built-in resistance against changes despite wide variations in temperatures.

Recently, the concept of fragile/strong glasses gained biological significance since Walters (2004) was able to use it to calculate the molecular mobility associated with the degradation of dry anhydrobiotes. She predicted that for a water content $0.07 \text{ g} \cdot \text{g}^{-1}$, molecular rearrangement may still occur at cryogenic temperatures, thereby refuting the idea that cryogenic temperatures stops all biological reactions. According to such predictions, it was further shown that several accessions of lettuce seeds could not be safely stored in liquid nitrogen for as long periods as anticipated without significant loss of viability (Walters et al. 2004). The authors concluded that near T_g , biological glasses of dry seeds have intermediate fragility. However, both the presence of high collapse temperature (Buitink et al. 2000c, Murthy et al. 2003) and the moderate temperature dependence in molecular mobility between T_c and T_g (Buitink et al. 2000c) suggest that biological glasses rather behave like strong glasses (i.e., they resist thermal degradation), which can also explain the absence of crystallization in biological tissues. Clearly, much more work is needed to understand the behavior of viscous intracellular liquids in anhydrobiotes submitted to drying.

7.5 Predictions of Longevity by Glass Parameters

Despite the fact that T_g is arbitrarily defined, it has long been considered as a key parameter that can explain properties and shelf-life of food products (Levine and Slade 1991, Roos 1995). By analogy, biologists have used T_g as a parameter to assess the storability of seeds and pollen. However, there is no apparent correlation between T_g and storability of both seeds and pollen (Hoekstra et al. 2005). Likewise, it is now increasingly accepted that the bio-preservative efficiency does not necessarily match the T_g (Lerbret et al. 2005). Studying the stability of proteins embedded in a series of binary glasses made of trehalose and glycerol, Cicerone and Soles (2004) were able to show that the glass conferring the maximum stability did not have the highest T_g . Glass transition temperature per se cannot fully predict the storage behavior of dry seeds and pollen using state-diagrams like those presented in Fig. 7.1. Kinetic studies on deterioration of dry seeds and pollens over long-term storage have shown that there exists an optimum water content of storage, below which life span decreases (reviewed in Walters 1998). Also, the value of this optimum water content decreases with increasing temperature (Fig. 7.1). Furthermore, the temperature dependence of this optimum water content is parallel and below T_g . Therefore, T_g values cannot predict the experimental observations of an optimum moisture and its dependence on temperature.

The significant correlation between molecular mobility and optimum storage conditions provides a strong argument that the characteristics of the glass

are of importance for the longevity in the dry state (Buitink et al. 2000b). Indeed, characteristics of the intracellular glassy matrix are modified below the water content corresponding to the moisture content at optimum storage conditions (Buitink and Leprince 2004). Whereas above the optimum, molecular mobility decreases with the loss of water as predicted by the glass theory, it increases again with further drying below the optimum water content for storage (Fig. 7.1B). The linear relationships between measurements of molecular mobility, assessed by EPR (Buitink et al. 2000c) (Fig. 7.1C) or derived from theoretical considerations (Walters 2004), enable the predictions of aging rates and therefore life span of seeds or pollens. These linear relationships have an important practical implication in long-term preservation. First, the extrapolation to low water contents and/or low temperatures allows for the prediction of life spans at storage conditions that do not allow practical determination of survival (Buitink et al. 2000b, Walters 2004). As such, it was confirmed that for cryopreservation, the samples should not be stored too dried at temperatures below 0°C (Buitink et al. 2000b). Also, indirect measurements of the structural stability of biological glasses suggest that even at cryogenic temperatures (far below T_g), some molecular mobility is still maintained (Walters 2004, Walters et al. 2004). This was recently confirmed by He et al. (2006) on sugar glasses; the authors reported water diffusion in amorphous matrices at temperatures down to -110°C . This molecular mobility may be related to the fact that glasses are metastable and, as such, their properties such as porosity are changing over time while slowly returning to equilibrium. This phenomenon, also called physical aging of glasses, appears to influence the kinetics of seed and pollen deterioration according to theoretical considerations (Walters 2004, Walters et al. 2005a). Actually, the mobility data shown in Fig. 7.1 are thought to be a reflection of the relaxation times describing the structural mobility of the glassy state. The observation that the Avrami equation, which describes the kinetics of crystallization (thus glassy relaxation), significantly fits the loss of viability of a wide range of species during storage (Walters et al. 2005a, 2005b) and is another indication of a putative relation between seed aging and the slow return to equilibrium of the glassy state, even at cryogenic temperatures.

While the specific properties of the glassy state can explain the effect of temperature and water content on aging rates, they cannot explain the large differences in storability at the species levels [ranging from 0.2 to 1 year in pollens (Hoekstra 2005) and from 10 to more than 500 years in seeds (Walters et al. 2005b)]. Despite the presence of an intracellular glass, are there any deteriorate reactions occurring storage that are not linked or are weakly linked to the glassy state?

There are some data in the literature that provide a beginning of an answer to this question. They pertain to the physical and chemical events occurring in storage lipids and membranes in the dry state. The highly significant

correlation between longevities of a wide range of plant anhydrobiotes and the average number of double bonds per polar lipids molecules led Hoekstra (2005) to suggest that deteriorative processes occurring in membranes are responsible for the loss of viability during storage, regardless of their T_g . Interestingly, for a particular seed or pollen species, the glassy properties are nevertheless able to predict the rate of these detrimental reactions leading to death in dependence to temperature and water content. This raises the question as to which extent the glassy state affects the rate of these deteriorative process occurring in the membranes. A weak but significant correlation was found between seed longevity (stored in the glassy state) and storage lipid content (Priestley 1996, Walters et al. 2005b). The presence of triacylglycerols has been associated with poor storage behavior (Priestley 1986, Walters 1998). Characteristically, they form a solid state between -10°C and 40°C . Interestingly, the rate of crystallization correlated with the rate of deterioration in *Cuphea carthagenensis* seeds, a species that exhibit an intermediate behavior (Crane et al. 2003, 2006). We do not know whether this process also affects the longevity of orthodox seeds. However, Crane et al. (2006) quote seed growers as saying that it might be the case for seeds with high oleic acid content. Also, changes in the physical behavior of triacylglycerols have been reported in dry aged soybean seeds (Vertucci 1992). Clearly, within the glassy state, lipids can still undergo physical changes, which might explain the loss of viability during dry storage.

One of the most widely accepted mechanisms leading to seed deterioration during storage is lipid peroxidation or oxidation due to the presence of reactive oxygen species, although direct evidence for a cause-effect relationship has yet to be secured (Walters 1998, Murthy et al 2003, Hoekstra 2005, Kranner and Birtic 2005). In food science, there is evidence that a glass can offer protection against oxygen-induced injury. For example, a glass made of sucrose, maltodextrin, and gelatine protects encapsulated oil from oxidation by decreasing the rate of oxygen permeation (Orlien et al. 2000). Generation of radicals in the glassy matrix by a hydrophilic azo-initiator did not accelerate the oxidation of the encapsulated oils, and the generated radicals were found to be immobilized in the matrix, whereas a lipophilic azo-initiator accelerated the oxidation of the oil (Orlien et al. 2000). However, these authors demonstrated that the diffusion of oxygen through the glassy matrix was the rate-determining step. Extrapolating to biological material, this study gives interesting insight into how glassy and nonglassy parameters interact to influence the shelf life. The work by Kranner and colleagues (reviewed in Kranner and Birtic 2005) constitutes an indirect evidence that oxygen diffuses within the dry intracellular glasses and might be responsible for the survival in the dry state. Another example is that of Yeh et al. (2005), who found an improvement of longevity of dry primed bitter melon seeds when they were stored under partial vacuum. However, it is not known how the characteristics

of biological glasses influence the permeability of oxygen to its targets that are embedded in the amorphous matrix. Permeability involves a thermodynamic factor (i.e., solubility) and a kinetic factor affected by molecular mobility (diffusion) (Gaudin et al. 2000, Dole et al. 2004). Diffusion and solubility of oxygen depend on the available free volume (Gaudin et al. 2000) or on the magnitude of volume not occupied by molecules (Andersen et al. 2000). These factors may well apply to intracellular glasses. However, the seed coat is also likely to play a significant role in controlling the diffusion of oxygen prior to its entry into glasses (Clerkx et al. 2004a). In this respect, it is noteworthy that there exist *Arabidopsis* mutants whose seeds have aberrant testa and exhibit a reduced longevity under laboratory conditions.

7.6 Putative Composition of Intracellular Glasses

When the concept of biological glasses was born, sugars were thought to take an important part in the composition and properties of the intracellular glassy matrix. This assumption was based on the observation that plant anhydrobiotes were known to accumulate large amounts of nonreducing sugars, predominantly sucrose and oligosaccharides, and on the knowledge that soluble sugars were known to be excellent glass formers. Although there are data providing correlative evidence that intracellular glasses are made of nonreducing sugars (Williams and Leopold 1989, Koster 1991, Buitink and Leprince 2004), they do not give information on to what extent the sugar molecules influence the biological and physicochemical properties of the intracellular glassy matrix. Several lines of evidence suggest that intracellular glasses are not made of sugar molecules alone. The state diagrams obtained from seeds and pollen are different from that of the corresponding carbohydrate mix (Buitink and Leprince 2004). Also, pregermination treatments that result in a decrease in oligosaccharides do not affect the state diagram of seeds of impatiens, pea, and bell pepper (Buitink et al. 2000a). Despite considerable changes in the sucrose/oligosaccharide contents, no change in the state diagram was observed for developing axes of soybean (Sun et al. 1994) and legume seeds (O. Leprince, unpublished data). In contrast, in sunflower cotyledons, maturation led to an upward shift in the T_g curve concomitantly with an increase in raffinose content (Lehner et al. 2006). Genetic analysis of seed longevity using *Arabidopsis* recombinant inbred line populations showed that a QTL affecting natural aging colocalized with a QTL for sucrose content but not with that for stachyose content (Clerkx et al. 2004b). However, the absence of a significant correlation between sugar content and seed longevity of more than 250 species casts doubts that carbohydrates play a primary and significant role in long-term stability afforded by the glassy state (Walters et al. 2005b). The idea

that nonreducing sugars are not the predominant components of intracellular glasses in plant anhydrobiotes is further reinforced by the observation that the physical properties of sugar glasses are quite different from those of biological glasses. Yet, considering that nonreducing sugars are highly abundant in seeds and that they will inevitably turn into the amorphous state during drying, their functional participation in the glassy state is expected.

Most likely, the predominant components to participate in the glassy matrix and determine for a large part its characteristics are proteins that are highly abundant in the cytoplasm. Evidence to suggest that proteins participate in the intracellular glass formation in anhydrobiotes is rather indirect (see Buitink and Leprince 2004 for references). We found that the temperature dependence of the molecular motion in biological glasses was more comparable to that of protein glasses rather than sugar glasses (Buitink et al. 2000c). In glasses made of poly(L)lysine (PolyLys), a highly hydrophilic synthetic polypeptide, the critical temperature T_c occurs at a temperature much higher than that of sugar glasses. However, it is noteworthy that the rotational motion in these protein glasses is almost twice as fast as in intracellular glasses (Buitink et al. 2000c). Because high molecular mobility in model glasses is generally associated with a loose hydrogen bonding network, it is suggested that the molecular packing of protein glasses is less dense than that for intracellular glasses. Thus, additional molecules likely contribute to the increase of the local density in intracellular glasses. Indeed, adding sugars to protein glasses results in a decrease in molecular mobility and increase in density (Buitink and Leprince 2004, Cicerone and Soles 2004).

Although the nature of proteins involved in the intracellular glasses are unknown, late embryogenesis abundant (LEA) proteins, a family of small hydrophilic proteins that are heat soluble, could be prime candidates (Wolkers et al. 1998b 2001, Boudet 2005). Because during maturation, seeds and pollen accumulate both nonreducing sugars and LEA proteins (Cumming 1999, Hoekstra et al. 2001), the current hypothesis is that both type of molecules interact together to form a glassy state. Sinniah et al. (1998) found that accumulation of both sugars and an unknown 58-kDa heat-soluble protein was correlated with potential longevity that was assessed at 15% moisture and 40°C. Using an LEA protein purified from *Typha latifolia* pollen (Wolkers et al. 2001) and from *Medicago truncatula* seeds (Boudet 2005), the molecular structure of a sucrose glass was found to change with the addition of an LEA protein in the direction of that found for intracellular glasses. The hydrogen bonding strength of the sucrose/LEA mixture is higher than that of a sucrose glass alone. However, a mixture made of sucrose and poly(L)lysine, a highly hydrophilic polypeptide, exhibited a higher hydrogen bonding strength than mixtures of LEA proteins/sucrose and this low-molecular density was achieved at lower concentrations of poly(L)lysine in the sugar matrix than LEA proteins (Wolkers et al. 1998b, 2001). Although this observation

indicates that LEA proteins do not exert a special effect on hydrogen bonding, their high abundance in all cellular compartments of dry anhydrobiotes still makes them likely components of intracellular glasses. Considering that LEAs proteins form an heterogeneous class (Wise and Tunnacliffe 2004), whether there are specific LEA proteins involved in determining glass properties in dry anhydrobiotes remains to be addressed. Predictions arising from bioinformatics suggest that LEA proteins could form a sort of cytoskeleton in the dry state, thereby providing mechanical strength to the cytoplasm (Wise and Tunnacliffe 2004). More work is needed to assess the implication of LEA in biological glasses or elucidate whether other types of proteins play a role in the glass formation. It should be noted that the advantage of a combination of sugars and LEA proteins in the glass mixture would be that both types of molecules exhibit several and distinct functions, all important for the preservation of structural functionality.

Other solutes might participate to the formation of intracellular glasses. In this respect, it is noteworthy that MgCl_2 and small polyhydroxy compounds (e.g., glycerol) can interact together on drying to form a self-polymerizing protectant with coordinate polymer chains of high T_g (MacFarlane et al. 2002). Also, a small amount of citrate is able to affect the glass properties of a sucrose glass by increasing T_g and strengthening the hydrogen bonding network, resulting in denser amorphous matrix (Kets et al. 2004). Considering that the cytosol and organelles matrices are made of a complex mixture of sugars, proteins, amino acids, ions, and salts, it can be speculated that intracellular glasses are likely to be made up of a mixture of sugars and proteins, which in turn interact with additional cytoplasmic molecules.

7.7 Concluding Remarks

Glass formation is not a mechanism that initially confers the tolerance to desiccation during drying, but the formation of intracellular glasses is indispensable to survive the dry state. The resulting characteristic high viscosity slows down deteriorative reactions but does not stop them totally. This can be observed by characterizing the properties of intracellular glasses rather than measuring T_g . These properties vary according the temperature and moisture content, even below T_g . Roughly 40° to -60°C above T_g , there is a second kinetic phenomenon that is also dependent on temperature and water content. This parameter, which can be assessed by measuring T_c , should be taken into account when one extrapolates aging kinetics measured under conditions far above T_g to those leading to the glass formation. In other words, care should be taken by using observations made under accelerated aging (>85% RH and temperatures >40°C; i.e., close to T_c) to predict the storage behavior below T_g .

Although comparisons of model sugar glasses with intracellular glasses should be considered with caution, it is hypothesized that sugar molecules could play a role in filling the large molecular voids between larger molecules (i.e., proteins), which in turn provide moieties for hydrogen bonding, whereas water fills the smaller voids close to both the carbohydrate chains and the proteins. These processes result in an increase of the density of glasses, allowing less molecular mobility in the matrix and greater stability.

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8 DNA Structure and Seed Desiccation Tolerance

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8.1 Introduction

Desiccation tolerance, which is a relatively rare event in whole plants, is of obvious importance in seeds. The majority of plant species produce seeds that are “orthodox” with regard to their ability to withstand storage at low moisture content. For these species, desiccation is sometimes considered to be a prerequisite for the completion of the life cycle, and they have evolved various mechanisms to maintain the integrity of their molecular structures in seed embryos under conditions of desiccation. This chapter describes the molecular events linked to the maintenance of DNA integrity in seeds at their entry into a desiccation-tolerant state and on early rehydration.

In the orthodox seed, the events that take place in the embryo during dehydration on the mother plant follow a sequence that can be linked to the decreasing water content of the seed and its embryo (Osborne 2000). DNA replication is the first metabolic process to be arrested (Brunori 1967), and this provides evidence for the high sensitivity of DNA synthesis to desiccation (Fig. 8.1). After shutdown of DNA replication embryo cells in most species arrest in the G_1 stage of cell cycle (Bino et al. 1993), so that DNA repair, which is responsible for genome integrity, is likely to be very efficient on activation. Cells arrested in G_1 show a 2C DNA content. Whether the extent of DNA duplication during dehydration plays a part in determining desiccation tolerance is still unknown, but dry seeds with a very low percentage of 4C nuclear values in the embryo cells belong to the most highly desiccation-tolerant species (Boubriak et al. 1997). Arrest of transcription and translation follow the cessation of DNA replication and, by the time the seed moisture content has fallen to 14% to 16%, all ATP-requiring activity has ceased and the seed embryo has become desiccation tolerant.

The loss of seed moisture during maturation drying may be crucial for conformational changes in the DNA. With the lowering of water activity conformational changes in DNA sequences can lead to the formation of supercoils, cruciforms, or anisomorphic or slipped structures. The attainment of stable secondary DNA structures that are resistant to degradation *in vivo* at low water potential is a likely accompaniment to desiccation tolerance (Osborne and Boubriak 1994).

The overall stability of the genome is always under threat of endonuclease nicking and digestion as well as the effects of free radical action on DNA.

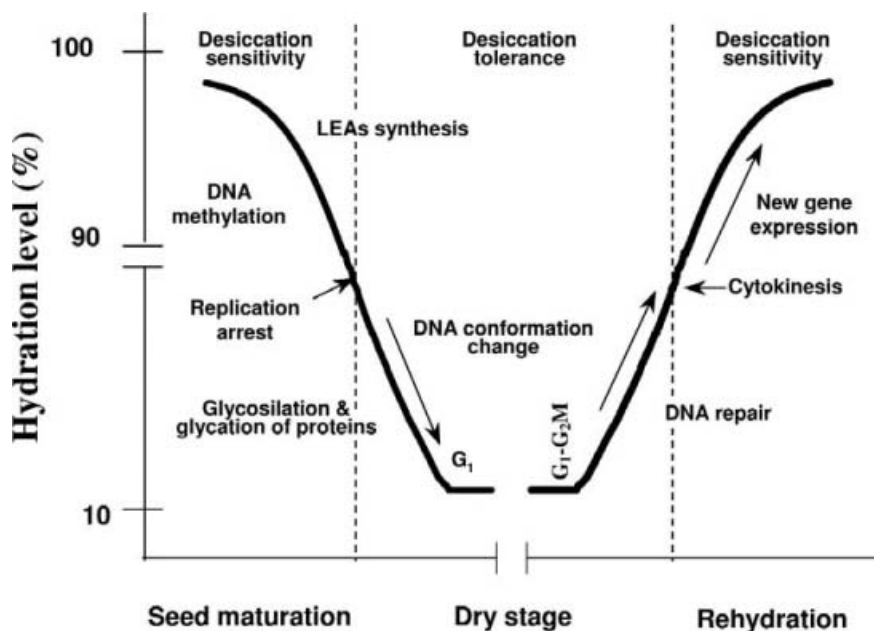


Fig. 8.1 Progression of molecular events in orthodox seed embryo during water restriction on maturation and renewal of free water on rehydration in relation to desiccation tolerance.

Water stress increases the formation of reactive oxygen species (ROS), resulting not only in lipid peroxidation and protein denaturation but also in nucleic acid damage (Hansen et al. 2006). Even in the dry state, progressive loss of DNA integrity and increasing levels of random-sized small-molecular-weight fragments, without a loss of total DNA, occur (Cheah and Osborne 1978). Whereas hydrated cells have the potential for immediate repair of DNA damage, the nuclei of dry cells will accumulate DNA lesions until such time that rehydration takes place and DNA repair enzymes can again operate. Thus, the stability of the DNA during desiccation and the efficiency of DNA repair on rehydration can be seen as integral components of the total desiccation tolerance mechanism in seeds.

Seed desiccation tolerance can be tested only by activation/survival of embryo cells on rehydration. The greater the desiccation protection afforded to the cell during the dehydration process, the greater is the DNA integrity retained during dry stage, and the less is the demand that is made on the early transcription capacity of DNA for the resynthesis of the main components of the cell. If a highly efficient DNA repair capacity is preserved, partial fragmentation of DNA may not be a serious impediment to desiccation survival, but loss of DNA repair function can be seen as a prelude to cell death

(Boubriak et al. 1997). The relationship between desiccation tolerance, DNA fragmentation, efficiency of DNA repair, and programmed cell death will be also described in this chapter.

8.2 Desiccation and Maintaining the Integrity of the Genome

8.2.1 Hydration-Determined DNA Conformations

Water is the most important and active of all biological molecules, and it is now clear that liquid water is not a “bit player” in the theatre of life, but it is a “star performer.” In bulk liquid water, each water molecule not only forms up to four tetrahedrally directed hydrogen bonds with neighbouring water molecules, but each molecule can also establish a dipole and induce dipole interaction with other molecules. The atoms of biomolecules can replace any or all of the links around each water molecule, which affects the structuring of adjacent water molecules and biomolecular groups. Water molecules can be bound to the surface of biomolecules by stronger or weaker forces than their average bulk-phase interaction (Chaplin 2006).

In the nuclear DNA, water molecules are intrinsic to the phosphate backbone of the fully hydrated B-form of the DNA double helix that exists in most living cells. In fact, B-DNA is stabilized by a spine of hydration running down the minor groove in the DNA structure (Drew et al. 1981, Dickerson et al. 1982). Withdrawal of this water leads to a change from the B-form of DNA to the A-form, with an increase in the number of base pairs per turn from 10.5 to 11. With a further increase of desiccation, DNA transforms into the Z-form with 12 base pairs per turn (Dickerson et al. 1982). Further dehydration will result in the least hydrated D-DNA (favoured by excess counterions that shield the DNA phosphate charges), which has a very narrow minor groove with a string of alternating water and counterions distributed along its edge and which has only 8 base pairs per turn (Fuller et al. 2004). Recent studies using infrared spectroscopy have showed that dehydration of supercoiled plasmid DNA induces a transition from a B-conformation in solution to a mixed conformation in the dried state. Changes in vibration of the bases upon drying suggest a change to the A conformation, whereas vibrations from the phosphate moieties suggest the A- or C-form. Vibration changes in the ribose ring suggest adoption of a C conformation (Choosakoonkriang et al. 2003). Molecular conformations assumed by DNA in fibers are highly reproducible and fully reversible (Fuller et al. 2004).

When the DNA is dehydrated, its length changes and this depends on the mode of dehydration (Vollenweider et al. 1978). Of the different types of dehydration tested, the freeze-dried DNA form is the longest, whereas dehydration

Table 8.1 Contour lengths and axial rises per nucleotide pair of various forms of dehydrated phage λ DNA as determined by electron microscopy.

DNA Preparations	Absolute DNA Length,* μm	Average Axial Rise/ Nucleotide Pair [†] , \AA	Relative Length
Freeze drying	16.48 ± 0.21	3.43 ± 0.08	100
Ethidium bromide intercalation and freeze drying	22.86 ± 0.29	4.75 ± 0.11	139
Dehydration in methanol (90%)	15.94 ± 0.20	3.31 ± 0.08	97
Dehydration in ethanol (90%)	15.24 ± 0.21	3.17 ± 0.08	92
	13.93 ± 0.19	2.90 ± 0.07	85
	12.42 ± 0.19	2.58 ± 0.07	75

* Confidence intervals calculated at 0.99 confidence level and including magnification error and the DNA contour length error. Confidence intervals arising from the standard deviations of measurements of the carbon grating replica squares, the latex circumferences, and the DNA contour length were geometrically added.

[†] Confidence intervals calculated at 0.99 confidence level and including errors of magnification, DNA contour length, and $\pi\text{RFII-to-}\lambda\text{cI26}$ ratio.

After Vollenweider et al. 1978.

in methanol or in ethanol results in progressively shorter molecules. These measured lengths of freeze-dried, methanol-dehydrated, and (shortest) ethanol-dehydrated forms correspond to the axial rise per nucleotide pair in the B-, C-, and A-form of DNA, respectively (Table 8.1).

In the crystal structures of different DNA forms, the free oxygen atoms of adjacent phosphate groups along the polynucleotide chain in B-DNA are found at least 6.6 \AA apart and individually hydrated, but they are as close as 5.3 \AA in A-DNA and 4.4 \AA in Z-DNA. In fact, this more economical hydration in A- and Z-DNA compared with B-DNA is the underlying cause of B-form to A-form and B-form to Z-form transitions (Saenger et al. 1986). The most relevant difference between A-, B-, and Z-DNA lies in the conformation of the sugar ring, which is related to the distances between the phosphate groups at the 3' and 5' ends of each nucleotide and to the displacement of base pairs relative to their helical axis. When the hydration schemes of the phosphate groups in the different DNA conformations are compared, it is evident that in B-DNA the average oxygen-oxygen separation of about 6.6 to 8.1 \AA is so large that each phosphate group can be surrounded by its own sphere of water molecules. With a cutoff of 3.5 \AA for O-O water distances, one or two water molecules cannot bridge adjacent phosphates. In contrast in A- and Z-DNA, the oxygen atoms of adjacent phosphate groups are so close together that they can be bridged by one or two water molecules (Saenger et al. 1986).

Different DNA sequences have different abilities to bind water molecules. In the B-form of DNA, an AT base pair binds one more water molecule than a GC base pair and another water molecule is present at each thymidine,

Table 8.2 Statistics of water binding to free phosphate oxygen atoms O(1), O(2) in different DNA fragments.

Sequence	I	II	III	IV	V	VI	VII
Helix type	A	A	B	B	B	Z	Z
Number of water molecules bound to O(1), O(2)	40	34	22	35	32	37	35
Forming bidentate intraphosphate bridges	1	0	1	2	2	1	1
Forming monodentate interactions with O(1), O(2)	31	30	21	33	30	34	31
In one-water interphosphate bridges	7	4	0	0	0	5	3
In two-water interphosphate bridges	4	1	1	2	1	2	2
Interphosphate gaps bridged by one water, %	58	33	0	0	0	63	38

After Saenger et al. 1986.

which confers additional stabilization of the B conformation. Indeed, the thymidine-bound water molecule is believed to be first to be lost on dehydration of DNA (Saenger et al. 1986). At the same water potential, dodecamers containing AATT sequences favor the B conformation, octamers with TATA sequences favor the A-form, and hexamers enriched in GC favor the Z-form. Therefore, DNA conformation at different water potentials will be determined by combined physical and chemical interactive forces (Hagerman 1990), and the hydration-driven transitions between these conformations are fully reversible (Fuller et al. 2004), which should be of a crucial importance for DNA structures in living cells (Table 8.2).

To date, we do not have enough evidence of the existence of differently dehydrated DNA forms in vivo in plants and in seeds. But conformational changes from the B-form to the A-form and the Z-form take place during dehydration in prokaryotes (Mohr et al. 1991, Setlow 1992, Kabakov and Poverenny 1993, Hayes et al. 2000, Shirkey et al. 2003) and during differentiation in specialized eukaryotic cells (Nordheim et al. 1986, Kabakov and Poverenny 1993). In bacteria, such DNA conformational changes are facilitated by certain groups of DNA-binding proteins and they are fully reversible on rehydration (Setlow et al. 1992, Setlow 2006).

8.2.2 DNA-Binding Proteins at Desiccation

8.2.2.1 Conformational DNA Changes and SASP Proteins. The first direct evidence that DNA-binding proteins can play a role in conformational changes in DNA was obtained in the study of the DNA binding capabilities of small acid-soluble spore proteins (SASPs) of *Bacillus subtilis*. Analyses using circular dichroism and Fourier-transform infrared spectroscopy have demonstrated that binding of α/β -type SASPs to DNA in vitro causes a structural

change in DNA, from the B to the A conformation (Mohr et al. 1991). The binding of SASPs to DNA was saturated at a protein/DNA ratio (wt/wt) of 4:1 to 5:1, which is approximately one SASP per 4 base pairs. The relative affinity of α/β -type SASPs for different DNAs was $\text{poly(dG)} \cdot \text{poly(dC)} > \text{poly(dG-dC)} \cdot \text{poly(dG-dC)} > \text{plasmid pUC19} > \text{poly(dA-dT)} \cdot \text{poly(dA-dT)}$, with $\text{poly(dA)} \cdot \text{poly(dT)}$ giving no detectable binding (Setlow et al. 1992). This order of α/β -type SASP-DNA affinities parallels the facility with which the DNA adopts an A-like conformation. As predicted by the conformational change in DNA, binding of SASPs to relaxed, but covalently closed, plasmid DNA results in the introduction of a large number of negative supercoils. Associated with the conformational change in DNA brought about by SASP binding is a change in its photochemistry such that ultraviolet irradiation does not generate pyrimidine dimers, but rather thymine-thymine adducts termed spore photoproducts. The later two properties of DNA complexed with α/β -type SASPs in vitro are similar to those of DNA in vivo in dormant spores of *Bacillus* sp. in which plasmid DNA has a much higher number of negative supercoils than plasmid in growing cells, and ultraviolet irradiation produces only spore photoproducts in the dry state, and only pyrimidine dimers in germinating and growing cells (Setlow 1992), which is an additional confirmation of the presence of A-type DNA in dormant *Bacillus* spores. Further studies of DNA-SASP complexes have been attempted using circular dichroism spectroscopy (Hayes et al. 2000), but detailed structures for these were not possible to establish because of the large cooperative nature of binding, as well as a tendency of the nucleoprotein complexes to form massive aggregates. The best available, to date, three-dimensional structure for the helical complex between DNA and SspC (a characteristic member of the α/β -type SASP family) was obtained using cryoelectron microscopy by a modified version of the interactive helical real space reconstruction method (Frenkiel-Krispin et al. 2004), which combines single-particle and helical reconstruction techniques (Fig. 8.2). The SspC protein is found to fully coat the DNA, forming distinct protruding domains, and to modify DNA structure such that it adopts a 3.18-nm pitch, which is closer to a canonical B-form, than to the A-form. A novel mode of nucleoprotein organisation is identified in which tight packaging of DNA-SspC filaments is mediated by interdigitation of protein domains from adjacent helices (Frenkiel-Krispin et al. 2004). By sequestering altered DNA molecules and promoting DNA dehydration this dense assembly of filaments is proposed to enhance and complement the DNA protection afforded by saturation of DNA with the SASP.

Acquiring a different DNA form from the canonical B-form at desiccation can provide a number of advantages to living cells. Altering the DNA structure by α/β -type SASPs prevents DNA damage in spores of *Bacillus* species arising from different environmental factors, such as UV and heat, desiccation, and variety of toxic chemicals (Nicholson et al. 2000, Setlow 2006). In

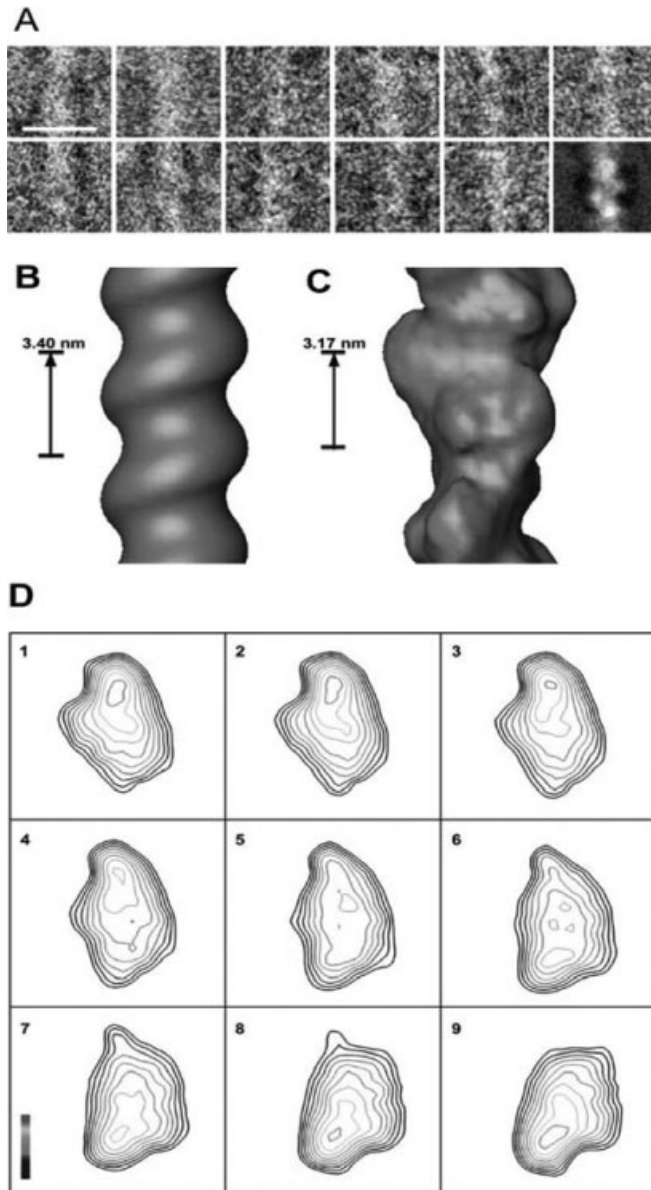


Fig. 8.2 DNA-SpC 3D reconstruction. (A) Gallery of raw “particles” (helix sections) that have been aligned by rotation and translation. The last frame shows the average of all aligned particles belonging to one representative class from the reconstruction. (B, C) Surface representation of the initial model (B) and final reconstruction (C). (D) Slices through the reconstruction (perpendicular to the helical axes). Consecutive slices are separated by 0.235 nm and cover one helical subunit. After Frenken-Krispin et al, 2004. For color detail, please see color plate section.

contrast, mutant spores lacking most α/β SASPs are very sensitive to these factors. Analysis of DNA extracted from heated (85° C, 30 minutes) and untreated wild-type spores of *Bacillus* sp. as well as untreated α/β SASP-lacking spores revealed very few single-strand breaks (less than 1 per 20 kb) in the DNA, whereas DNA from heated α/β -SASP-lacking spores had more than 10 single-strand breaks per 20 kb (Fairhead et al. 1993). Quite probably, this can be explained by a reduced rate of DNA depurination—an initial cause for the formation of single-strand breaks at elevated temperatures. A role of SASPs in protecting DNA against depurination was predicted and demonstrated in experiments in vitro, where these proteins reduce the rate of DNA depurination at least 20-fold (Fairhead et al. 1993). This protective action of SASPs against depurination was also confirmed in vivo by correlating the survival rate of spores with the number of abasic sites in the DNA (Setlow and Setlow 1994).

The protective role of SASPs is not limited to protection from DNA-damaging factors. For example, the saturation of spore DNA with SASP proteins dramatically increases spore wet-heat resistance. Exactly how wet heat kills spores is not clear, although it is not through DNA damage (Setlow 2006).

Alterations in DNA structure caused, at desiccation of cells, by DNA-binding proteins not only provide protection for the DNA molecules itself, but they can also affect other DNA-related biochemical processes, such as replication and repair. It is known that sequence-dependent structural variations in double DNA helices may interfere with the fidelity of DNA replication and transcription by altering base pair conformations. Adopting an A-like conformation on water exclusion from DNA will attenuate the structural variability and thus enhance replication fidelity (Mayer and Timsit 2001). A tight, toroid-like assembly of the nucleoprotein also facilitates DNA repair including error-free repair of double-strand breaks (Weller et al. 2002). This is possibly because, within the rigid matrix, free DNA ends generated by double-strand breaks are kept firmly together as a result of restricted diffusion, allowing for error-free repair through template-independent nonhomologous end joining.

Conformational changes in DNA and protection against desiccation are not solely dependent on the binding of α/β -type SASPs to DNA. Very recently a role for another class of organic molecules in *Bacillus* spores was demonstrated. It was shown that the presence of dipicolinic acid (DPA) in the spore core can contribute significantly to the protection of the DNA from desiccation (Setlow et al. 2006). DPA in spores can lower the water content in the spore core by replacing some core water interactions with DPA. DPA also contributes significantly to the protection of DNA in *Bacillus* spores from many types of damage.

It is not yet proved that mechanisms of DNA protection from desiccation by small acid-soluble proteins operate in plants or that conformational change in DNA contributes to desiccation tolerance mechanisms in orthodox seeds.

But SASPs have been found in the nuclei of dry rye embryos (Osborne 2000), and conformational changes in DNA that alter the recognition of specific base sequence domains by enzymes may well explain the different fragmentation patterns of DNA in the desiccation-tolerant and -intolerant states of rye embryos (Osborne and Boubriak 1994, 2000a, 2000b, Chiatante et al. 2002). What is obvious is that a number of components synthesized during desiccation may act as DNA protecting factors, stabilising nucleoproteins during drying.

8.2.2.2 LEA Proteins and DNA Binding. Between the various macromolecules such as sugars and proteins involved in stabilizing cellular structures during drying, late embryogenesis abundant proteins (LEAs) are the most promising candidates for the DNA protective role in dried embryos. These proteins are synthesized anew in the late stages of embryogenesis in seed embryos during formation drying on the mother plant (Blackman et al. 1991). The role of LEAs as the molecules responsible for desiccation tolerance mechanisms in seed embryo cells (Wise 2003, Wise and Tunnacliffe 2004, Goyal et al. 2005, Boudet et al. 2006, Manfre et al. 2006), as well as many other cell types (Browne et al. 2004, Kikawada et al. 2006), is now well established despite still very limited knowledge of the actual mechanisms underlying their action. LEAs constitute a set of proteins (35 proteins in *Arabidopsis thaliana* [Illing et al. 2005]) that show common physiochemical properties, such as high hydrophilicity and high content of small amino acid residues e.g., serine, alanine, and glycine (Campos et al. 2006). LEA proteins found in different plant species, are now divided into at least six different groups defined on the basis of their expression pattern and amino acid sequence (Wise 2003). Proton NMR intensity and differential scanning calorimetry measurements have been carried out on some of the LEAs to characterise their water- and ion-binding capacity. It was shown that typical unstructured LEA protein, ERD10, not only has a very high hydration capacity, but it can also bind a large amount of charged solute ions (Tompá et al. 2006). Such physicochemical properties of this protein will allow it simultaneously to retain water in drying cells and prevent an adverse increase in ionic strength, thus acting to prevent protein denaturation during dehydration stress.

Furthermore, Fourier transform infrared spectroscopy of certain other LEA proteins (MtPM25 and MtEm6) showed that they exhibit only a certain degree of order in the hydrated state but adopt α helices and β sheet conformations and become much more structured during drying (Boudet et al. 2006). It is quite possible that, at different hydration levels, mechanisms of protection by LEAs will change and that they can protect different components of the cell at high and low hydration.

Reversible phosphorylation of proteins is an important mechanism by which cells regulate their reaction to external stimuli, including dehydration. It was

shown that dehydration-induced proteins are often transiently phosphorylated during dehydration, but phosphorylation patterns differ for different representatives of this class (Rohrig et al. 2006). The coincidence of phosphorylation sites with predicted coiled-coil regions led to the idea that phosphorylation of LEAs can influence the stability of coiled-coil interactions of LEA proteins with themselves and possibly with other proteins.

Until recently it was not clear whether LEAs only protect protein components of cells (by changing protein conformation [Shih et al. 2004] and acting as a novel form of molecular chaperone or “molecular shield” by preventing protein aggregates formation during water stress [Goyal et al. 2005]) or whether they also have DNA binding functions similar to those of SASPs.

One of the first pieces of evidence of possible protection, by LEAs, of the nuclear DNA from desiccation was described for *Solanum chacoense*, which is extremely resistant to drought. It was shown that in protein DS2, which is structurally similar to LEAs, the carboxyl terminus predicts an α helix and contains a bipartite nuclear targeting sequence motif (Silhavy et al. 1995). More recently, a nuclear localization of one of the LEAs (belonging to dehydrin group) was shown for potato cells (Chiatante et al. 2002). In nuclei of potato cells, growing under low water potential, conformational modifications of the chromatin were detected and stabilization of chromatin by nuclear LEA molecule-salvation action was predicted.

A recent comprehensive study of all groups of LEA proteins, using a number of statistically based bioinformatics tools, has shown that representatives of at least two groups (group 2 and 3 LEA proteins) are involved in desiccation resistance via a role in DNA binding (Wise 2003). These proteins emphasize α -helical structures and, at a large scale, filaments and such LEAs are natively unstructured but become structured under stress conditions (Wise 2003, Wise and Tunnacliffe 2004). Such feature, together with their nuclear localization, predicts DNA protective function for these LEAs. Direct evidence of this may soon be available by using a novel and versatile method for detection of the position and dynamic nature of protein–DNA interaction, namely unzipping force analysis of protein association (Koch et al. 2002). Furthermore, DNA binding of LEA regulatory proteins (Ditzer and Bartels 2006) to “hydration fingerprints” of given DNA sequences (Chaplin 2006) at different desiccation regimes may aid our understanding, not only of the protection but also of the regulation of desiccation tolerance at the DNA level.

8.2.2.3 Nuclear DNA Changes in Dry Stored Seed. There is no precise moisture content that can be attributed to the “dry state” of seed. It is generally accepted that a seed can be said to be in the “dry state” when the moisture content of the embryos falls below 10%. The cytoplasm of dry embryo enters into a glassy state in which molecular movement is strictly limited (Williams

and Leopold 1989, Buitink et al. 2000, Buitink and Leprince 2004), and synthetic events of all kinds are excluded (Bewley 1979). But even at this level of hydration, “non–energy-requiring” processes can take place such as activation of nucleases and generation of free radicals, which can affect the integrity of DNA molecules in the dry state.

The first evidence of DNA changes in stored seed was obtained as early as 1933 when Navashin had shown accumulation of chromosome aberrations during dry storage of seed (Navashin 1933). The extent of these changes during storage was dependent upon the condition of the seed when it was shed from the parental plant.

The maternal history during seed maturation is partly determined by environmental factors (such as humidity, light, and temperature) and partly by the genetic constitution of seed, so that when seed is shed, embryo vigor is already a predetermined variable. Even so, the original quality of mature fresh seed can be dramatically affected by storage conditions, especially by levels of humidity, temperature, and oxygen content (Osborne et al. 2002).

Even under optimal (dry) storage conditions, the progressive loss of DNA integrity to produce increasing levels of random-sized small DNA fragments is evident in recalcitrant seeds (Cheah and Osborne 1978). This is a result of the accumulation of single- and double-strand DNA breaks in embryo cells—the main type of DNA lesions in the dry state (Osborne 1982). However, no DNA is lost from the nuclei, as was demonstrated by microdensitometric analysis of stained nuclei in rye embryo squashes (Boubriak et al. 2000a, 2000b). The amount of DNA per nucleus remains constant in embryo cells even though it becomes highly fragmented in seeds that have been dead for 7 years or longer.

Oxidative DNA damage is also evident in DNA from dry embryos, because the dehydration conditions can promote accumulation of reactive oxygen species in seeds (Leprince et al. 1994, Franca et al. 2006). Under normal hydration conditions, free radicals would be scavenged by antioxidant defense systems, however, in the dry state these mechanisms might not function (Franca et al. 2006). Alternative mechanisms of DNA protection from oxidative damage may exist in desiccation-tolerant tissues, as was shown for the cyanobacterium *Nostoc commune*. Dormant cells of *Nostoc* have shown quite low levels of the main types of oxidative DNA damage (8-hydroxyguanine, 8-hydroxyadenine, and 5-hydroxyuracil) even after prolonged desiccation periods (Shirkey et al. 2003). For plant tissues, protection from oxidative damage at dehydration has recently been attributed to specific LEA proteins (Mowla et al. 2006).

Despite heavy fragmentation of the DNA in the dead seeds, the nucleosome structure of chromatin remains almost unchanged in such embryo cells. This has been demonstrated in experiments involving DNase I digestion of isolated nuclei from dead and fresh dry embryos (Table 8.3). Both produced similar mononucleosome and oligonucleosome ladders not found in untreated

Table 8.3 Nucleosome contents of fresh and dead rye embryos held in dry and accelerated aged conditions.

Seed Sample	Moisture Content	Viable Embryos	Dead Embryos
Dry	10%	ND	ND
Imbibed, 20 h	>90%	ND	13.3 ± 2.3
Accelerated aged, 5 days	13%	ND	ND
Accelerated aged, 15 days	14%	5.9 ± 1.0	ND
Accelerated aged, 19 days	14%	8.7 ± 2.0	ND
Accelerated aged, 19 days; stored dry, 70 days	14%, 10%	10.3 ± 2.0	-
Accelerated aged, 19 days; imbibed, 20 hours	14%, 90%	21.1 ± 4.8	15.6 ± 3.0

ND, nondetectable levels.

dry embryos (Cheah and Osborne 1977). This is a good evidence for random DNase cleavage of DNA molecules in the dry state rather than specific cleavage of sequences in H1 linker regions.

The situation regarding DNA fragmentation is dramatically different when the hydration conditions for seed storage are not ideal. In experiments with rye, when accelerated aging conditions were tested, site-specific DNA cleavage and nucleosome accumulation became evident after 15 days of treatment (Boubriak et al. 2000a, 2000b, Osborne and Boubriak 2000).

Direct analysis of the accumulation of nucleosomes has revealed that accelerated aging conditions (leading to an increase of embryo moisture content to 14%) will activate a nucleosome generated nuclease in viable seed, but not in the dead seed, although this specific nuclease becomes active at full hydration even in dead embryos, when the seeds are placed in water (Osborne and Boubriak 2000). It is very clear that, with respect to the integrity of DNA, the conditions of accelerated aging for viable rye do not reflect those taking place in the dry state and the accelerated aging procedure cannot be used as a quick method to study changes linked to the slow cell death of seeds that are held in the dry state (Osborne and Boubriak 2000).

In both animal and plant cells, nucleosome accumulation is a hallmark of programmed cell death (PCD) (Greenberg 1996, Joselin et al. 2006), and it is quite possible that seed embryos may have mechanisms to trigger PCD, if the conditions for the maintenance of DNA integrity on storage are not favorable. Oxidant and antioxidant signaling can play a major role in the metabolic interface between plant stresses and responses to them in plants, including PCD (Laloi et al. 2004, Foyer and Noctor 2005, Laloi et al. 2006). A model was proposed that shows the relationship between “half-cell reduction potential” ($E_{\text{GSSG}/2\text{GSH}}$) and processes that eventually lead to DNA fragmentation (Kranter et al. 2006). This model suggests that an increase in $E_{\text{GSSG}/2\text{GSH}}$ during seed aging in a state of low hydration will have an impact on the

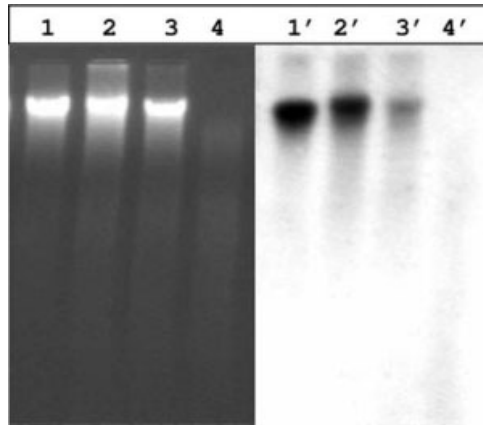


Fig. 8.3 DNA mobilities in neutral agarose gel fractionation (1 to 4) and telomere distribution shown by Southern hybridization (1' to 4') for embryos of dry stored rye seeds. Probe 5'-(CCCTAAA)₃-3' used for labeling. Lanes 1 and 1', high viability seed (98% germination); lanes 2 and 2', low viability seed (32% germination); lanes 3 and 3', nonviable seed (0% germination); lanes 4 and 4', accelerated aged (15 days) seed (0% germination).

various thiols involved in both initiation and execution of PCD in seed embryos.

The integrity of nuclear DNA is critical for fast and successful germination of seeds and depends on the level of DNA damage accumulated, both before and during storage. Since the 1940s, it has been shown that the ends of chromosomes perform a special function in maintaining the linear structure of DNA (McClintock 1941), and it is clear that loss of the DNA sequences that make up these chromosome ends is, in many cases, associated with loss of DNA integrity and shortened life spans (Kilian et al. 1995, Riha and Shippen 2003, Fajkus et al. 2005).

The first experiments to study the constancy of telomere repeat length in the DNA of embryos in stored dry seeds was carried out on wheat (Bucholc and Buchowicz 1992). It was shown that telomeres in wheat embryos become considerably shortened as seeds age over a period of 6 to 12 months. Because seeds aged for both time periods showed 94% germination, this suggests that the loss of telomere sequence must precede the loss of viability and seeds stored for longer will lose more telomeres and require more telomere replacement (Osborne and Boubriak 2002).

We have found that a progressive loss of telomeric sequences occurs from high-molecular-weight DNA with an increasing appearance in low-molecular-weight DNA as the periods of storage in the dry state were extended so that seed germination was reduced to 98%, 32%, and 0% (Fig. 8.3). Telomere

distribution in DNA would appear to follow the general pattern of random DNA fragmentation that occurs in the embryos of seeds stored in the dry state, but there are also indications of an overall telomere loss from DNA as a consequence of storage (Boubriak et al. 2003).

Whether there is an equal loss of telomere repeats due to random DNA fragmentation in the dry state (10% moisture content) as due to a specific nucleosome pathway (at 14% moisture content) leading to DNA disintegration is not yet known. However, it is quite likely that the loss of DNA integrity that occurs in seed embryos, whether it be from aging dry, aging under high moisture content, or under stress conditions generally will always exhibit some release of telomere sequences from the nuclear chromatin, which can then be isolated as extrachromosomal DNA (Osborne and Boubriak 2002).

8.2.3 DNA Repair and Desiccation Tolerance

Dehydration and desiccation represent one of the most frequent and severe challenges to all living cells. This is not surprising, because desiccation conditions may be due to multiple environmental factors such as extreme temperatures, alkaline pH, exposure to sunlight, high salinity, etc. Several lines of evidence suggest that the same DNA repair mechanisms that are usually associated with gamma- and UV-irradiation or chemically induced DNA damage are also important in protecting cells from desiccation (Wilson et al. 2004).

A number of investigations on prokaryotic communities exposed to arid conditions (e.g., in the hot desert of Tatouine [Chanal et al. 2006] or Sonoran Desert [Rainey et al. 2005]) has shown a correlation between tolerance to desiccation and tolerance of radiation. The recovery of large numbers of extremely radiation-resistant bacteria from arid soils, which were not found in nonarid soils, provides further ecological support for the hypothesis that radioresistance is a consequence of the evolution of DNA repair systems that protect cells against commonly encountered stresses such as desiccation (Rainey et al. 2005).

Direct evidence for a role for DNA repair mechanism in desiccation tolerance was obtained in UV-sensitive mutants of *Escherichia coli* (Asada et al. 1979). An *E. coli* mutant defective in nucleotide excision repair and recombination (*uvrA*⁻ *recA*⁻) was more sensitive to drying to a water activity of 0.53 or below than the parent strain. The drying condition used led to the accumulation of DNA strand breaks in bacteria, and the extent of DNA strand breakage was observed to be similar for every strain tested (mutants and wild-type). But desiccation-resistant strains repaired damaged DNA during postdrying incubation, whereas desiccation-sensitive strains were unable to do so (Asada et al. 1980).

Nucleotide excision repair and, possibly, recombination repair are not the only mechanisms responsible for desiccation tolerance in prokaryotes. Recently, the role of endonucleases ExoA and Nfo that can repair apurinic/apyrimidinic sites and strand breaks in *Bacillus subtilis* DNA was demonstrated (Salas-Pacheco et al. 2005). It was confirmed that these nucleases are additional factors that protect bacterial spores from DNA damage accumulated during spore dormancy.

A novel mechanism of DNA repair of double-strand breaks that can accumulate at desiccation was shown for the extremely radioresistant bacteria *Deinococcus radiodurans*. This organism can survive in harsh conditions in arid desert environments that will shatter its genome into hundreds of short DNA fragments (Mattimore and Battista 1996). However, these fragments will be reassembled into a functional genome using a previously unknown two-stage DNA repair process called “extended synthesis-dependent strand annealing” (ESDSA) (Zahradka et al. 2006). In ESDSA, chromosomal fragments (at least two copies of the genome are needed) with overlapping homologies are used both as primers and as a templates for massive synthesis (dependent on DNA polymerase I) of complementary single strands. Newly synthesized complementary single-stranded extensions become “sticky ends” that anneal with high precision forming long linear, double-stranded DNA intermediates. These intermediates require recA-dependent crossover to mature into circular chromosomes that comprise a patchwork of numerous DNA blocks (Zahradka et al. 2006). Amazingly, this very complicated process of resurrection from “clinical death” due to desiccation is a high fidelity process and, therefore, allows bacteria to survive even after extreme desiccation.

8.2.4 DNA Repair Mechanisms in Plants

Plant cells, when hydrated, are constantly coping with damage to their DNA that is generated endogenously or through the action of environmental stressors (including drought-desiccation, changing temperatures, radiation, and chemical mutagens). Endogenous damage, known as “spontaneous DNA damage” (Britt 1999), is produced by reactive metabolites such as oxygen free radicals, hydroxyl radicals, superperoxide, and nitric oxide, as well as defects in the normal process of DNA metabolism (Tuteja et al. 2001). All types of DNA damage can be repaired through the different repair pathways that exist in plants, and these include photoreactivation of UV photoproducts, base excision repair (BER), nucleotide excision repair (NER), replicative lesion bypass, double-strand break repair, and mismatch repair (Britt 1999, Bray and West 2005).

All repair systems except photoreactivation, which is specific to UV-induced DNA lesions, can be involved to a certain extent in the repair of desiccation-induced damage in plants. Essential DNA maintenance in plants is carried out

by BER, which can eliminate single-base damage including deamination, methylation, oxidation, or base loss. Several specific glycosylases have been described for plants including uracil-DNA glycosylase (Talpaert-Borle and Liuzzi 1982), 3-methyladenine-specific DNA glycosylase (Santerre and Britt 1994), and formamidopyrimidine DNA glycosylase (Gao and Murphy 2001, Morales-Ruiz et al. 2003). Some of the glycosylases such as DEMETER are crucial for seed viability (Choi et al. 2002), because they are also involved in gene self-imprinting by allele-specific demethylation (Gehring et al. 2006, Takeda and Paszkowski 2006). Some of the plant DNA glycosylases are known to have a secondary activity and to be involved in repair of abasic (AP) sites by cutting the DNA backbone 3' to the damaged site (Garcia-Ortiz et al. 2001). AP endonucleases that can cut the sugar-phosphate backbone 5' of the abasic site also have been described for plants (Babiychuk et al. 1994, Yupsanis et al. 2004). Recently, homologues of the other main BER enzyme, DNA polymerase β , have been found in plants (Sarkar et al. 2004, Mori et al. 2005). There is still no evidence for DNA ligase III, which we would expect to be involved in BER, and no homologue of this enzyme is found in the current plant genome databases (Bray and West 2005), but other ligase complexes (Yamamoto et al. 2004) may substitute for this enzyme.

The most versatile system for dealing with the DNA damage accumulated on desiccation and seed aging is NER. This system can repair different types of damage because it recognises conformational changes to the DNA duplex rather than a specific type of DNA damage. NER eliminates a 24-32 oligonucleotide containing the damaged site, followed by resynthesis of the missing fragment using the opposite DNA strand as a template.

NER was first identified in plants as early as the 1970s when removal of UV-induced pyrimidine dimers was demonstrated in carrot protoplasts and plant seedlings (Howland 1975, Soyfer and Cieminis 1977). This repair system has been shown to operate in different plant tissues including pollen (Jackson and Linskens 1978, Boubriak and Grodzinsky 1985) and seeds (Osborne 1984), and it is able to remove DNA damage caused by UV- and gamma-irradiation, chemical mutagens, heavy metals, and desiccation (Jackson and Linskens 1980, Grodzinsky and Boubriak 1985, Boubriak et al. 1997). Genes essential for the NER pathway were initially characterised by EMS mutagenesis, and a number of *uvr* and *uvh* complementation groups that show hypersensitivity to UV-C have been defined (Jiang et al. 1997).

DNA damage detection in NER in plants can occur, as in other eukaryotes, by two different systems, depending on the transcriptional activity of the damaged region: general genomic ("global") repair, or transcription-coupled repair (Bray and West 2005). NER in eukaryotes is catalyzed by six multiprotein complexes, and homologues of many of the proteins in these complexes have been found in plants. Proteins of the damage recognition complex XPC/hHR23B have been found in *Arabidopsis* (Schultz and Quatrano 1997,

Liang et al. 2006), and this is modulated by another *Arabidopsis* protein, AtCEN2 (Molinier et al. 2004, Liang et al. 2006). Helicases from the transcription factor complex TFIIH (homologues of XPB and XPD) are also found in this model plant (Ribeiro et al. 1998, Liu et al. 2003, Morgante et al. 2005), and the function of AtGTF2H2 and AtXPD in NER has been confirmed (Vonarx et al. 2006). After unwinding of DNA by helicases and recruitment of the RPA and XPA complex, 5' endonucleases ERCC1/XPF and 3' endonuclease XPG nick the damaged strand. Two different RPA homologues have been found both in *Arabidopsis* and rice, and at least one of these is principally involved in DNA repair (Ishibashi et al. 2005).

The 5' endonuclease is probably the best-described NER enzyme in plants. Both subunits of this endonuclease, Atercc1 (Hefner et al. 2003, Dubest et al. 2004) and AtRAD1 (Gallego et al. 2000, Dubest et al. 2002), have been identified in *Arabidopsis* and it has been shown that the *uvh1* mutant, which is hypersensitive to UV-C, lacks the AtRAD1 protein (Liu et al. 2000). Involvement of the 5' endonuclease in plant NER has now been confirmed for *Arabidopsis* by an in vitro repair synthesis assay (Li et al. 2002). Another 3' endonuclease (XPG) required for excision repair of UV damage has been identified using the *Arabidopsis uvh3* mutant, and the full product of this gene (AtRAD2) has been analyzed (Liu et al. 2001). *Arabidopsis* also possesses DNA polymerases α and Δ as well as DNA ligase I, which is essential for completion of NER (Taylor et al. 1998, Gomez and Vazquez-Ramos 2003, Sunderland et al. 2004, Kunz et al. 2005, Garcia et al. 2006).

Double-strand DNA break repair, which is crucial for seed germination after the desiccation stage, can be achieved by two different recombinational mechanisms: homologous recombination (HR) and nonhomologous end joining (NHEJ). Three models for HR, developed mainly using the yeast model, have been described (Krogh and Symington 2004, Cahill et al. 2006). These include synthesis-dependent strand annealing (SDSA), double-strand break repair (DSBR), and a single-strand annealing model (SSA). All three HR pathways operate in plants and many of the protein complexes involved in recombination are common to different HR mechanisms (Schuermann et al. 2005, Bleuyard et al. 2006). Analysis of DSB repair in *Arabidopsis* revealed that recombination frequencies vary greatly, depending on the origin of the donor and recipient sequences. Whereas homology presented in allelic or ectopic positions is hardly used for repair, the use of homologous sequences near the break site is frequent. An SSA mechanism that leads to sequence deletion between direct repeats is particularly efficient (Puchta 2005). The conserved SDSA process, resulting in conversions without crossover, is also prominent and seems to be important for the evolution of tandemly arranged genes (e.g., drug resistance genes) (Orel and Puchta 2003, Puchta 2005). Further analysis of DSB repair revealed that DSB repair mechanisms are developmentally regulated, and the proportion of breaks repaired via HR substantially decreases

as the plants mature (Boyko et al. 2006a). Moreover, the level of occurrence of double-strand breaks was also different among different plant tissues, with the highest level found in roots (Boyko et al. 2006b).

Somatic and meiotic recombination pathways in plants have common molecular machinery and are catalyzed by RPA and proteins of the RAD52 epistasis group (Puchta 2005, Bleuyard et al. 2006). *Arabidopsis* HR null mutants such as AtRAD51, AtMRE11, and AtMND1 show extensive chromosome fragmentation, dependent on the function of SPO11, and are also completely sterile (Bleuyard et al. 2004, Kerzendorfer et al. 2006). Despite this, an *Rad51* knockout in *Arabidopsis* shows only slight hypersensitivity to gamma radiation (Bleuyard et al. 2005), which suggests a minor role for HR in the repair of double-strand breaks in plants with the hypersensitivity of *rad50* and *mre11* knockouts only reflecting the roles of these genes in NHEJ (Bray and West 2005). However, recent studies have shown that other proteins of the RecA/RAD51 family are also involved in HR (OsRADA in rice and AtRADA in *Arabidopsis*) (Ishibashi et al. 2006) and, in a tobacco experimental system, double-strand breaks can be repaired by HR and NHER with similar frequencies (Pacher et al. 2006). In contrast to the recent findings, it is still generally thought that plants primarily utilize an NHEJ to repair DSB (Friesner and Britt 2003, West et al. 2004).

The first molecular data on end-joining in plants came from experiments on maize and *Arabidopsis* and analysis of footprints left behind after transposable element excision (Rinehart et al. 1997). In NHEJ, the initial recognition of DSB is mediated by a complex of Ku70 and Ku80, which protects the ends from exonucleases and juxtaposes the two ends of the DSB, independent of sequence homology (West et al. 2002). After processing of the ends and removal of overhanging nonhomologous DNA "tails" by 5' endonuclease (Dubest et al. 2004), ligation is catalyzed by a DNA ligase IV/XRCC4 complex (West et al. 2000). The role of Ku70 and Ku80 in NHEJ was confirmed using *Arabidopsis* mutants in these genes, which are sensitive to agents that induce strand breaks in DNA, such as gamma-rays, bleomycin, etc. (West et al. 2002). Despite being sensitive to DSB-inducing agents, these *Arabidopsis* mutants can still grow on relatively high concentrations of bleomycin, which suggests the presence of alternative repair pathway(s) (Bray and West 2005, Bleuyard et al. 2006). It was shown that, in Ku mutants, end-joining showed greater use of microhomologies, whereas in the pathway that is independent of both Ku and Mre11 genes, end-joining was associated with the insertion of filler DNA sequences (Heacock et al. 2004). Such filler DNA insertion suggests that illegitimate end-joining should involve synthesis-dependent annealing, which is different from SDSA-mediated HR (Puchta 2005).

An important feature of Ku proteins recently described in plants is their role as a negative regulator of telomerase function in cells (Gallego et al. 2003, Gallego and White 2005). A role in telomere maintenance and the protection

of chromosome ends is described for another component of the recombination machinery, Rad50 protein (Vannier et al. 2006). The absence of AtRad50 leads to rapid shortening of a subpopulation of chromosome ends and subsequently chromosome-end fusions lacking telomere repeats. It was suggested that the protective action of Rad50 proteins in *Arabidopsis* is in confining recombination to sister chromatids and thus avoiding end-to-end interactions (Vannier et al. 2006).

These observations provide a fascinating link between the repair of stress-related DNA DSB and restoration of telomere ends. The interconnection between different repair pathways that exist in plants (Schuermann et al. 2005) and their capacity to deal with both spontaneous and induced DNA damage can dramatically affect plant genomes and their survival rate under influences of the environmental stressors including desiccation.

8.2.5 DNA Repair Efficiency on Early Rehydration

If the mature seed, at the desiccation-tolerant state, is stored in a way that does not impair the ability to germinate, then the events of rehydration from dry condition follow an essentially similar pathway of nuclear reactivation for all the embryos that have been studied in details (Osborne et al. 2002). The first event on the imbibition in water is the physical hydration of the cytoplasm, which will occur even in dead embryos (Hallam et al. 1973) and will be completed within first hour of imbibition (Obroucheva and Antipova 2004). In living seeds, further uptake of water proceeds continuously, and this is accompanied by transcription and protein synthesis.

In rye embryos, transcription of all heterogeneous RNA classes (4S, 5S, 18S 25S, and 31S) has been shown by electrophoresis of newly synthesized radiolabeled nucleic acids within 60 minutes (Sen et al. 1975). Incorporation of labeled amino acids into proteins is evident even earlier, 15 minutes after imbibition, confirming the concept of long-lived mRNA coding for protein synthesis in the first hours of germination (Osborne et al. 1977).

Repair of DNA by a dark excision repair mechanism also commences at once (Osborne et al. 1981), but DNA replication in G_1 and transition to 4C nuclear values in G_2 is delayed and may not be evident until many hours after embryos become fully hydrated. It is not yet known if the start of replication requires a particular physical state of hydration. Some metabolically active embryos, with moisture content as high as 40%, remain dormant. A typical example is *A. fatua*, in which dormant embryos can defer replication of their DNA and progression to G_2 for months or years before initiating cell cycling and attendant germination (Osborne et al. 2002). The full explanation for the control factors that hold embryos in a dormant homeostatic but metabolically very active state is still not available, and such investigations are of special

interest, because throughout dormancy, imbibed seeds are still desiccation tolerant and can be dried back without any loss of their viability.

The clue to survival of dormant embryos during the whole period of imbibition in water, is their ability to maintain a functional DNA repair system (Elder and Osborne 1993). As was shown in experiments with *A. fatua*, repair of gamma-induced single- and double-strand DNA breaks is similarly efficient in both dormant and nondormant imbibed seeds. Repair of irradiation damage is fully completed within 2 hours in both in dormant and nondormant *A. fatua* seeds and a similar pattern for repair is evident for other orthodox seeds such as rye (Elder and Osborne 1993, Osborne and Boubriak 1994). Importantly, this high-molecular-weight repaired DNA is stable to desiccation as long as the seed embryo remains dormant or the germinating seed is still in a desiccation-tolerant stage of germination (up to 6 hours in rye embryos) (Boubriak et al. 1997). The crucial role of DNA repair competence in desiccation tolerance was confirmed in experiments where the irradiated dormant embryos were imbibed in the presence of inhibitors of DNA polymerase α and DNA polymerase β enzymes essential for dark repair in plant cells (Boubriak and Grodzinsky 1986). In the presence of DNA repair inhibitors, only partial repair of gamma-induced damage was achieved and the still-damaged DNA suffered further fragmentation on drying back (Boubriak et al. 1997). Also it was shown that in hydrated nondormant rye, at a stage when desiccation tolerance is lost, embryo cells still repair radiation-induced damage, but the repaired DNA is unstable to desiccation and cannot be re-repaired when water is again made available. This failure to re-repair on rehydration appears to be absolutely critical to the embryo's survival and germination success (Boubriak et al. 1997, Osborne and Boubriak 1997).

It has been demonstrated in a number of laboratories that the enzymes of the DNA repair pathways belong to the group of proteins that are relatively unstable in dry embryos. DNA polymerases and DNA ligases essential for DNA repair lose function with time. Unless the activities of the enzymes of the DNA repair complex have remained sufficiently functional before germination is fully initiated by the presence of water, the embryo cells will fail to recover a functional genome and the seed dies (Yamaguchi et al. 1978, Elder et al. 1987, Vazquez et al. 1991, Coello et al. 1992). Indeed, we have known for many years that stored seeds take progressively longer to germinate and eventually will not germinate at all. The maintenance of operational DNA repair systems is therefore always an essential requirement for the success of seed survival in the dry state.

A separate issue for the germination of stored seeds is their ability to restore their telomere repeats. For seeds, we currently have little idea of how stable the telomere-associated proteins remain once in the different tissues of the dry seed, nor do we have much information on the early events that occur in embryo telomere termini on rehydration. What is known is that rye seeds that

have considerably depleted their telomeres during dry storage for up to 12 months will again acquire newly synthesized telomere sequences, and this repair process is completed within 3 hours of imbibition (Bucholc and Buchowicz 1992, Boubriak et al. 2003). A special case of DNA repair was suggested, and it was speculated that the low-molecular-weight extrachromosomal DNA, isolated as telomere-sequence-rich fragments, could be the possible source of new telomeric repeats for the imbibing wheat chromosomal DNA (Bucholc and Buchowicz 1992, 1995).

That telomere repeats increase when seed embryos germinate has been observed in barley and other plants (Kilian et al. 1995, McKnight et al. 2002). Telomerase activity, although low or undetectable in vegetative plant parts, has been detected at low levels in immature *Arabidopsis* seeds, in barley embryos (Heller et al. 1996), and particularly in the seedlings and root tips of certain other germinating seeds (e.g., *Silene*) (Riha et al. 1998). The association of telomere synthesis with telomerase activity in seeds seems rather evident. However, when telomerase activity is lacking, telomeres can be amplified by alternative mechanisms (Osborne and Boubriak 2002). In an *Arabidopsis* TERT mutant, which lacks the catalytic subunit of telomerase, end-to-end fusion of critically shortened telomeres is mediated by nonhomologous end-joining (Heacock et al. 2004). An important function of the NHEJ machinery in telomere maintenance was recently highlighted, describing how NHEJ and the telomere complex have evolved to maintain genome stability in plants (Riha et al. 2006).

A detailed description of all the molecular repair events that occur during seed transition from the dry state to germination and the contributions of the different repair pathways in repair of fragmented DNA and restoration of telomere ends in stored seeds is still not available, but there is no doubt that excision repair and systems involved in repair of double-strand DNA breaks and telomere restoration all have critical roles (Osborne and Boubriak 2002, Osborne et al. 2002, da Costa-Nunes et al. 2006).

8.3 Evolutionary Adaptations of Seeds and DNA Repair

Multiple strategies of adaptation of seeds to harsh desiccation conditions, especially of those in deserts, have evolved. Many seeds and fruits of the common species occurring in desert habitats have mucilaginous coats, including those from the Asteraceae, Cruciferae, and Plantaginaceae, and other families (Gutterman and Ginott 1994, Gutterman and Shem-Tov 1997, Boeken et al. 2004). The mucilage that covers the seeds or fruits gives them many ecological benefits, especially in extreme desert conditions, including hydrochory, endozoochory, ombrohydrochory, and the option of adhesion to the soil surface by means of mucilage after they are dispersed and wetted (Gutterman

1994, 2002, Boeken et al. 2004). However, the mucilaginous pellicle may play an even more important role in desiccation tolerance by enhancing dew absorption and maintaining a higher hydration level of the embryo.

Comparisons of the water-holding capacity of seeds with or without their pellicles showed that periods of hydration were extended after dew deposition if the pellicle was present. It was suggested that the value of the pellicle at low water availability is the provision of an enhanced opportunity for metabolic events in the embryo including DNA repair and maintenance of genomic integrity and that it is important for sustained viability in the seed bank (Huang et al. 2004). We have now shown that in *Artemisia sphaerocephala*, which is well adapted to survive in the Gobi, pellicle mucilage that forms on swelling provides sufficient moisture for repeated and cumulative periods of opportunistic DNA repair whenever night conditions cause dew condensation. We have confirmed that DNA repair occurs when pellicles are intact, but not if pellicles are removed (unpublished data). We believe that the opportunistic and cumulative DNA repair facility is an example of a valuable evolutionary adaptation and is important for the survival of many plants particularly in arid lands where rainfall is limited or unpredictable.

8.3.1 *Recalcitrant Seeds and DNA Repair*

Orthodox seeds are generally tolerant to water loss—often to 5% or less of their fully hydrated state—and they can be held in the desiccation-tolerant state. In contrast the seeds of many tropical and subtropical species do not acquire desiccation tolerance at the time of shedding. Those of *Avicennia marina* (mangrove), for example, will die if on dehydration the hydration levels falls below 50% (Berjak et al. 1984, Peran et al. 2004). The absence of one or more of the relatively extensive suite of mechanisms present in orthodox seeds is held to underlie the desiccation sensitivity of recalcitrant seed types (Pammenter and Berjak 1999).

It is generally thought that a continuous state of metabolic activity is the factor that characterises the desiccation sensitivity of most plant tissues and propagules (Pammenter and Berjak 1999, Berjak and Pammenter 2001). We have questioned whether the desiccation sensitivity of embryos of *A. marina* can be linked to continuous operation of the cell cycle, with replication of DNA that continues unabated during embryo maturation and germination, and whether DNA repair systems in such seeds are competent to repair the damage that occurs during dehydration. The question of whether repair of fragmented DNA still operates following dehydration was addressed by desiccating mangrove excised hypocotyl tips to 10%, 22%, and 46% water loss, then introducing DNA damage using gamma rays and immediately imbibing them to permit repair (Boubriak et al. 2000a, 2000b). These experiments were also

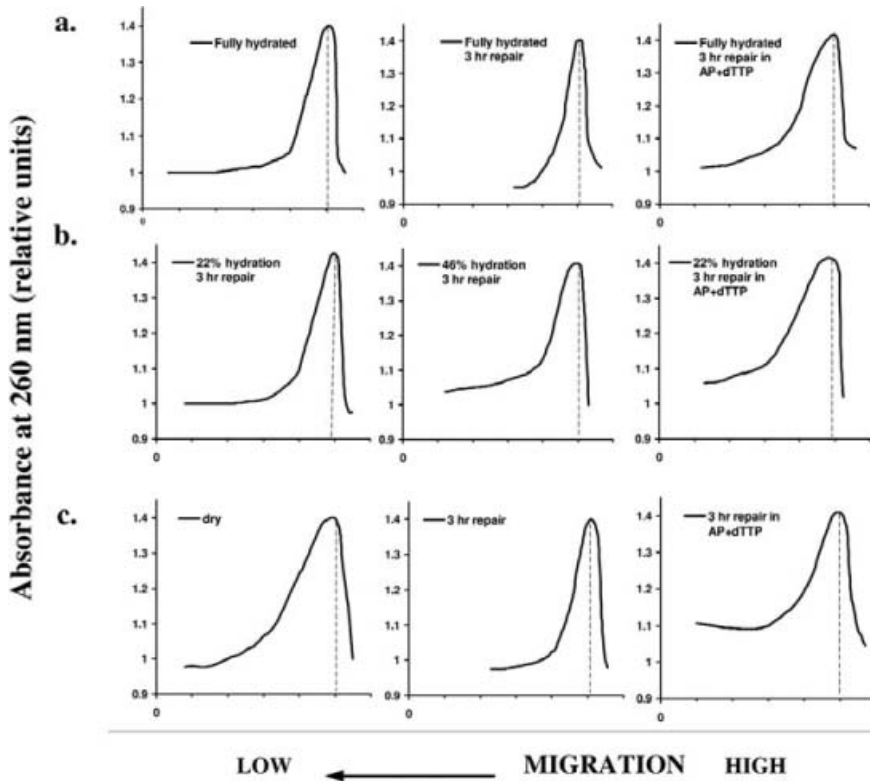


Fig. 8.4 Restoration of DNA integrity on rehydration of *Avicennia marina* (A, B) and *Secale cereale* seed (C). Comparison of molecular weight profiles of irradiated (750 Gy) embryos of freshly picked and differently dehydrated recalcitrant seed with dry orthodox seeds. Repair on imbibition into water for 3 hours with or without DNA repair inhibitors.

carried out in the presence or absence of inhibitors of essential dark repair enzymes (Fig. 8.4).

Electrophoresis of the extracted DNA shows that, whereas the fresh non-dehydrated tips can restore fragmented DNA to a high molecular weight, no repair had occurred in tips that were dehydrated by 22% and fragmentation was accentuated further when 46% dehydrated tips were imbibed or DNA inhibitors applied during the repair interval. It was established that DNA repair processes are severely curtailed or inactivated by water loss of only 22%. Further experiments on DNA fragmentation and nucleosome accumulation have shown that cell death is initiated in recalcitrant seeds of *A. marina* by water losses as little as 10% (Boubriak et al. 2000a, 2000b). It was concluded that labile DNA repair together with possible lack of a stabilized dehydration

conformation of the DNA are important factors contributing to the lack of desiccation tolerance in recalcitrant seeds (Osborne et al. 2002).

8.3.2 *Maintenance of DNA Integrity and Priming*

The requirement for metabolic reactivation and germination is water. If the water potential is not enough to start full germination, seeds will still initiate certain biochemical processes that can allow progression of embryo cells up to the S_1 stage of the first cell cycle. The seed will undergo important processes of pregermination (Heydecker et al. 1973) including those crucial for maintaining their viability, such as DNA repair (Elder et al. 1987, Boubriak et al. 2001). This capability, which can lead to improvement of the seeds' performance in suboptimal conditions for germination, is now widely used by the seed industry when they perform advancement or "priming" of seed material, because it occurs when seed embryos are still desiccation tolerant and can be dried back without harm (Heydecker and Coolbear 1977).

Although it has been generally known for at least 25 years that embryos cease to be desiccation tolerant once root elongation and cell division in the embryo are actively taking place (Sen and Osborne 1974), the exact details of nuclear DNA synthesis and DNA replication during priming have been established for only a few species: leek, pepper, and tomato (Bray et al. 1989, Lanteri et al. 1993, Saracco et al. 1995, de Castro et al. 2000). Monitoring quantitative changes in nuclear DNA content and the relationship to the initiation of the first cell cycle in different embryo tissues studied has led to the identification of root meristem cells as the first to suffer from stress and the first to breach the stress tolerance barrier. Overprimed material is the first to show this symptom, which leads to selective cell death in the root meristem. It was ascertained for all the species studied that none of the priming treatments led to cell division although nuclear DNA content increased as priming progressed with the cells reaching 4C levels in the nuclei of root tip cells immediately behind the quiescent centre (Redfearn and Osborne 1997, Powell et al. 2000).

Of considerable relevance is the new finding that nuclear volume in all three species was always greater in primed material than in the unprimed controls. This enhanced size (as measured by nuclear diameter) is retained when the seed is dried back at the end of the priming process. In the overprimed material many of these nuclei, particularly in the root tip region, appear to be in a prophase or prometaphase condition, although none was in metaphase. It is very evident that priming causes a reorganization of nuclear chromatin as well as some DNA replication bringing the embryos to a stage that is then developmentally ahead of unprimed controls (Redfearn and Osborne 1997).

Clearly, it is not completion of S phase or the start of the assembly of the chromosomes on the spindle fibers that marks the boundary between safe

priming and overpriming. Some earlier process, taking place at the nuclear level, predisposes the embryo to a reduced competence to overcome stress when the seed is planted and to reduced viability when the seed is stored.

Nuclear damage was incurred increasingly as priming proceeded. This was shown by isolating DNA from the embryos and fractionating this DNA by different electrophoretic procedures. Even the dry, unprimed, seed embryos contain some DNA damage in terms of single- and double-strand DNA breaks (Elder and Osborne 1993), but all the primed samples assessed showed an increase in low-molecular-weight fragments above the unprimed samples (Boubriak et al. 2001). More important is the speed and efficiency of embryo samples to repair endogenous or any additional DNA damage that the embryos might incur.

To evaluate this, equal doses of gamma-irradiation was given to isolated embryos of differently primed sugar beet seeds. This means that each sample possessed its own endogenous DNA damage together with the additional single-strand DNA breaks resulting from irradiation. Using labeled thymidine in the imbibing media, the rate of thymidine incorporation into the DNA can be used to measure of the rate of DNA repair synthesis and hence the repair efficiency of each sample (Boubriak et al. 2001).

As expected, DNA repair efficiency increased with the extent of the seed priming of sugar beet (Fig. 8.5). Because all the unirradiated seeds showed improved germination over unprimed material, the DNA repair systems were clearly operating effectively in all primed material. The situation was quite different in the irradiated seeds. Whereas normal primed material incorporated labeled thymidine efficiently with increasing irradiation dose, indicating that DNA repair capability was maintained, incorporation was less in the embryos of overprimed seeds, indicating a reduced DNA repair capability. This means that the embryos of overprimed seeds have a recognizable limit to their DNA repair capability, which makes them vulnerable to incomplete repair when exposed to stresses subsequently imposed upon the germinating seed.

It seems likely that the reduced DNA repair capability in overprimed samples is the basic cause of the lowered storage capacity found in certain overprimed seeds. DNA repair enzymes are known to be relatively labile in the dry seed (Elder et al. 1987, Vazquez et al. 1991). It is not surprising, therefore, that the embryo that already has a limited capacity for repair at the start of the storage period would show an even less capacity to repair when the storage period is extended.

The cells most likely to be stress sensitive will be those closest to cell division, i.e., with 4C DNA levels. Accumulation of low-molecular-weight DNA fragments, particularly on dehydration, is typical for overprimed samples (which have the highest number of 4C nuclei). Additionally, these are the samples that also show a lowered DNA repair capability following irradiation. As a result, these particular cells are poor survivors among the overall cell

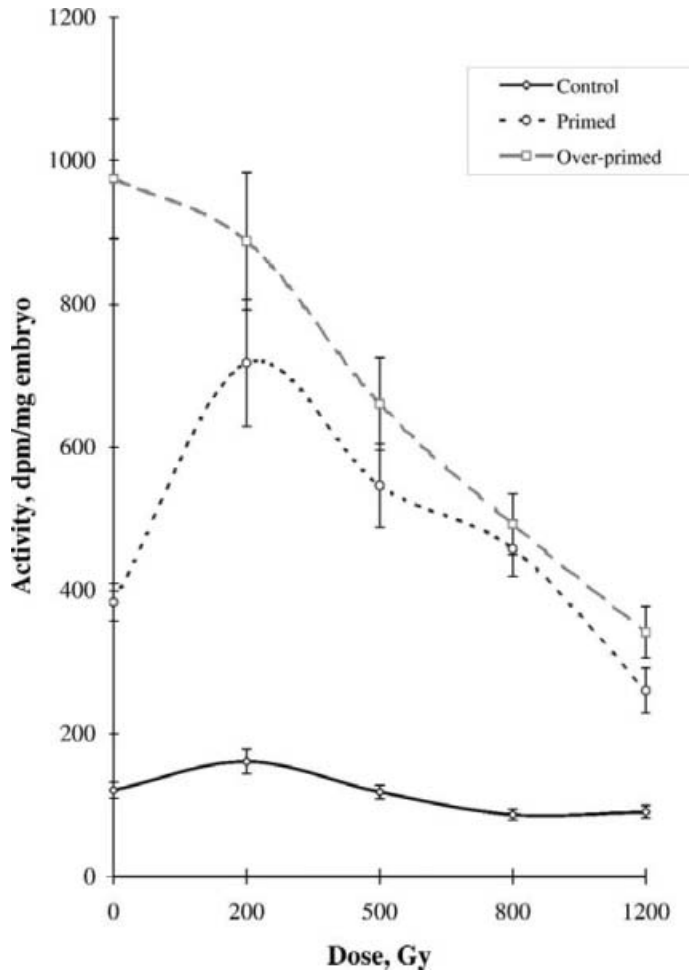


Fig. 8.5 Unscheduled DNA synthesis (measured by incorporation of H^3 -thymidine) in differently primed sugar-beets seeds after gamma-irradiation. Control, unprimed seed; primed, seed hydrated to 25% moisture content; overprimed, seed hydrated to 33% moisture content. For color detail, please see color plate section.

population and, in this sense, are the most sensitive to desiccation-induced death (Boubriak et al. 2001).

DNA excision repair activity is enhanced during any priming. Analyses of the extent of DNA fragmentation in differently primed material has shown that, during the drying stage that follows priming, DNA may again become damaged (showing the highest number of low molecular weight DNA in over-primed material), so further repair is needed on germination. In overprimed

seeds, DNA repair capability, which can accomplish the necessary repair resulting from drying, cannot fully repair further DNA damage resulting from irradiation. This means that any additional DNA damage (from any type of environmental stress including desiccation) by overprimed embryos is likely to be incompletely repaired on germination and that viability of the seeds and establishment of the seedlings in the field will be affected. The crucial role of DNA repair efficiency on priming for achieving restoration of the genome is so important that it was even proposed as a way of optimizing priming conditions using DNA repair tests (Boubriak et al. 2001). It is evident now that with increased knowledge of different DNA repair pathways in seeds on germination and their involvement in desiccation tolerance mechanisms, it will be possible to improve priming treatments to achieve maximum seed potential.

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9 Structural Dynamics and Desiccation Damage in Plant Reproductive Organs

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9.1 Introduction

Plant reproductive structures acquire considerable tolerance to dehydration during development and lose tolerance during germination. Desiccation tolerance mechanisms within plant reproductive structures allow tissues to survive considerable water loss, and contrast with drought tolerance mechanisms of roots, leaves, and stems of most plant species, which generally work to prevent water loss through osmotic adjustment (with exceptions). Embryos of some species (e.g., *Avicennia* and *Shorea*) remain sensitive to desiccation at shedding, although it can be argued that these embryos have germinated and are actually seedlings with properties reminiscent of vegetative structures (Berjak et al. 1989, Krishnapillay et al. 1998, Dickie and Pritchard 2002, Varghese et al. 2004). Surviving water loss buys time during dispersal to delay growth until conditions are amenable for plant establishment. Temperature and time are inextricably linked to desiccation stress because dried organisms must also survive cold, heat, or irregular rainfall patterns. Hence, survival in dry biological systems must also be considered in the context of the temperature and duration of the exposure. From this perspective, one may surmise a relationship between desiccation tolerance within plant reproductive structures and plant life history, mating strategies and gene flow. Greater incidence of sensitivity to desiccation is expected within reproductive structures of species originating in nearly constant environments (e.g., wetlands or tropical rainforests) or when dispersal distances are relatively short.

Plant reproductive structures provide ideal systems to study the impact of water loss on cellular systems. Cells lose water when water potential decreases below about -2 MPa. Unlike most vegetative tissues that are more adapted to retain water during slight stress (above -2 MPa), fully mature spores, pollen, and seeds have the capacity to survive -10 MPa or less when they are shed from the mother plant. Diverse physiologies in related taxa or among different organs (e.g., leaves, overwintering structures, pollen, and embryos) allow us to compare sensitivities among structures and identify primary lesions and cascading effects resulting from water loss. Cellular membranes are identified as a primary site of damage, as tensions from drying can shear membrane connections to the cell wall, cause loss of membrane material through vesiculation, or change intralamellar organization and fluidity (reviewed in Walters et al. 2002).

Cytoskeleton structure is also labile during drying and reassembly of microtubules is deficient in desiccation-sensitive systems (Mycock 1999, Berjak and Pammenter 2000, Berjak and Mycock 2004, Faria et al. 2005). Water deficit can induce production of reactive oxygen species (ROS) through signaling pathways, reactions that become uncoupled or changes in the redox state of the cells (Kranter et al. 2006, Walters et al. 2002). ROS attack and degrade all macromolecules (Halliwell and Gutteridge 1999), and kinetics of deterioration is a complex function of water status. Further damage comes from programmed cell death pathways, presumably activated to purge tissues from irreparably damaged cells (Kranter et al. 2006, Faria et al. 2005). Cellular derangements that result from desiccation were recently reviewed (Pammenter and Berjak 1999, Hoekstra et al. 2001, Walters et al. 2002). This chapter overviews some of these structural and biochemical changes and focuses on quantitation of damage in the context of the degree of stress and the time required for damage to occur. We borrow heavily from the pharmaceutical and food science literature where these questions apply to the stability of formulations as they are dried and stored. An important concept arising from these considerations is that desiccation-tolerant and -sensitive cells may not differ in the *types* of reactions that occur during dehydration; instead, the difference may lie in the slower *rates* of inevitable damaging reactions in tolerant cells.

9.2 Quantification of Desiccation Tolerance and Damage

Research during the past two decades brought recognition to the range of tolerances of desiccation found among organisms and to interactions among the stresses of desiccation, temperature and time. On one extreme is “absolute tolerance,” where an organism can survive the initial stress of complete water removal and then survive indefinitely in the dry state. As with absolute temperature, which is more a theoretical concept than an obtainable condition, “absolute tolerance” of desiccation is elusive. Many plant reproductive structures can survive the immediate effects of complete water loss (water content in equilibrium with 0% relative humidity [RH]), but they cannot survive forever in the dry state. Further, absolutely dry organisms appear to die faster than organisms that contain a modicum of water (e.g., Walters et al. 2005a). Seeds and pollen exhibiting this extreme, yet incomplete, level of tolerance are commonly referred to as “orthodox,” a name originally coined to describe the seed storage behavior of most crop species. Orthodox seed physiology is also common among nondomesticated species, especially those that are early successional (e.g., Dickie and Pritchard 2002).

In contrast to orthodox seeds, recalcitrant seeds and pollen do not survive complete drying and have only an ephemeral tolerance of water potentials as

low as -10 MPa. “Recalcitrance” is an anthropomorphic term originally used to describe seeds’ response to humans’ unsuccessful attempts to store them (Roberts 1973). The orthodox-recalcitrant distinction is an important dichotomy for seed banking endeavors because the requirement for water in desiccation-sensitive tissues precludes conventional storage conditions ($5\% \pm 2\%$ water at 5° or -18°C) (FAO/IPGRI 1994). In contexts other than conventional storage, the dichotomy between orthodox and recalcitrant may be somewhat arbitrary, as the degree of stress tolerance depends on level of maturity at harvest, postharvest treatments, and rate of exposure to water and temperature stress. Indeed, seeds from species classified as recalcitrant (Hong et al. 1998) exhibit a range of tolerances to water loss. Intraspecific variation occurs along latitude in species originating in temperate areas, presumably because longer growing seasons prolong the maturation period (Vertucci et al. 1995, Daws et al. 2004, 2006). Several laboratories report greater difficulties manipulating recalcitrant seeds that originate from tropical areas compared to those from temperate regions (e.g., Walters et al. 2007), perhaps because the embryos are immature or germinating and so do not have the full suite of protective mechanisms (Berjak et al. 1989, Vertucci and Farrant 1995).

In the early 1990s, the concept of “intermediate” physiology was introduced to describe seeds that survive desiccation but do not survive the combined effects of desiccation and low temperature (Ellis et al. 1990, 1991). The term has since been broadened to include seeds that do not tolerate as much drying as orthodox seeds and are damaged if equilibrated to 50% RH or less (about -90 MPa) (Hong and Ellis 1995, Dussert et al. 1999, Eira et al. 1999, Sacandé et al. 2000, Hor et al. 2005, Eira et al. 2006). Other intermediate seeds appear to tolerate the same degree of desiccation as orthodox seeds (e.g., *Cuphea*) but are damaged by interactions of water and crystallized triacylglycerols (Crane et al. 2003, 2006, Volk et al. 2006). The intermediate classification has also been used to describe seeds that survive the initial stresses of desiccation and low temperature but age rapidly. Clearly, it is difficult to separate the effects of desiccation, temperature, or time in reproductive structures classified as intermediate. Seeds with tolerances between those exhibited by orthodox and intermediate, or intermediate and recalcitrant are sometimes called “suborthodox” or “subrecalcitrant.”

The spectrum of desiccation tolerance and sensitivity encompassed by orthodox, suborthodox, intermediate, subrecalcitrant, and recalcitrant categories reveals the quantitative nature of protection and damage within reproductive structures in response to various stresses. Although quantitative, desiccation tolerance may not be a continuous feature where one may draw correlations between the amount of a particular protecting substance and the degree to which water stress can be tolerated. Damage from desiccation can result from a multitude of cellular disruptions and full tolerance may require a suite of

protective mechanisms expressed at the right time, in the right amount, within susceptible areas of the cell.

Desiccation tolerance is measured by survival as a function of water content (wc), water potential (Ψ), or RH if Ψ is less than -10 MPa. Stress versus survival curves are usually sigmoidal and tolerance is quantified by a critical water content or Ψ below which cells show symptoms of damage (Fig. 9.1). Alternatively, desiccation tolerance can be expressed as the water status at which 50% of the population of cells or organisms survives (e.g., w_{c50} , Ψ_{50} , or RH_{50} for water content, water potential, or RH, respectively). Water content is expressed on either a fresh or dry weight basis, the former providing a scale of 0 to 1 (pure water = 1) and the latter providing an infinite scale (pure water = ∞), so that small differences in water content are easier to discern. Water contents and water potential/RH are interconverted using isotherm relationships (Fig. 9.2). Isotherm properties vary with the chemical composition and structure of cells, with cells containing more high-molecular-weight or insoluble reserves having lower water contents for a specific water potential or RH (e.g., Vertucci and Roos 1990, Walters et al. 2001).

Expression of desiccation tolerance using both water content and water potential provides insights on the intensity of the stress and the resulting damage. For example, critical water contents of 0.42 and 0.77 g $H_2O \cdot g^{-1}$ dry mass in fully mature cotyledons and maturing embryonic axes, respectively, correspond to a critical water potential of -2 MPa in both tissues (Fig. 9.2).

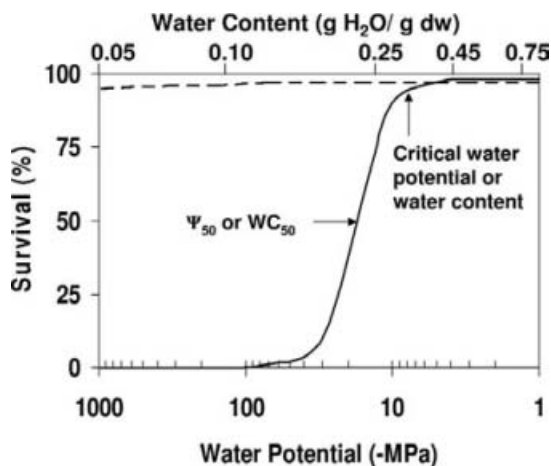


Fig. 9.1 Schematic diagram of the effect of water content or water potential on survival of a desiccation-tolerant (*dashed curve*) and desiccation-sensitive (*solid curve*) organism. The water content or water potential that marks loss of viability is considered critical. Sensitivity is also expressed as the amount of drying required to kill 50% of the population of cells or organisms. The schematics are typical of rapidly dried embryonic axes from orthodox (*dashed curve*) and recalcitrant (*solid curve*) seeds.

Cells subjected to -2 MPa experience drought stress rather than desiccation per se, and a critical water potential of -2 MPa would lead one to surmise that damage to embryonic tissue at this relatively mild stress resulted from metabolic dysfunction rather than structural collapse (Fig. 9.3). On the other hand, for the immature embryos described in Fig. 9.2, a critical water content of $0.77 \text{ g H}_2\text{O} \cdot \text{g}^{-1}$ dry mass corresponds to a critical water potential of about -5 MPa. Significant loss of water occurs in cells exposed to -5 MPa (see below), and so this critical water potential suggests that cells may be damaged from shrinkage (Fig. 9.3). A cell that survives drying to a water content of $0.10 \text{ g H}_2\text{O} \cdot \text{g}^{-1}$ dry mass is frequently considered desiccation-tolerant; however, this water content may correspond to a wide range of RH depending on the lipid content of cells. Assignment of desiccation tolerance based on survival to a single water content is usually misleading. It is also important to distinguish between the RH or water potential at which cells are dried and the RH or water potential that cells achieve. Water status of cells and the environment is only the same at equilibrium.

9.3 Kinetics Define Desiccation Damage

Desiccation stresses arise from both water removal from cells and time at low water potentials. Generally, damage is identified when cells die, indicating

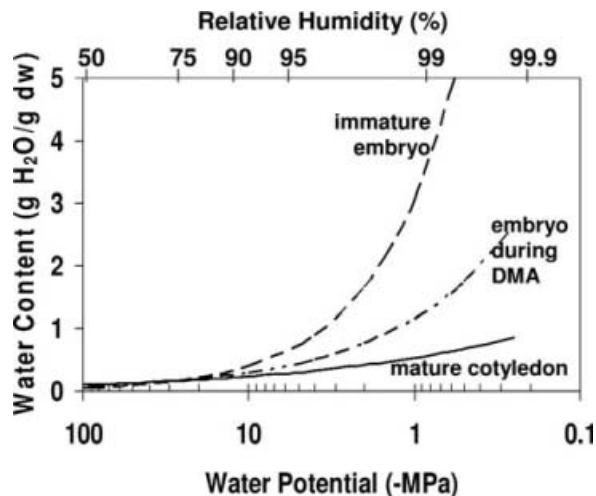


Fig. 9.2 Water sorption isotherms of embryonic tissues relating water content to water potential for water potential > -100 MPa. Isotherms are given for embryonic axes of *Aesculus hippocastanum* at the onset (dashed curve) and midway through (dot-dashed curve) dry matter accumulation and for mature pea cotyledons. These tissues contained about 15%, 40%, and 60% dry matter, respectively, at $Y = -0.5$ MPa. Data are from Farrant and Walters (1998) and Walters et al. (2001).

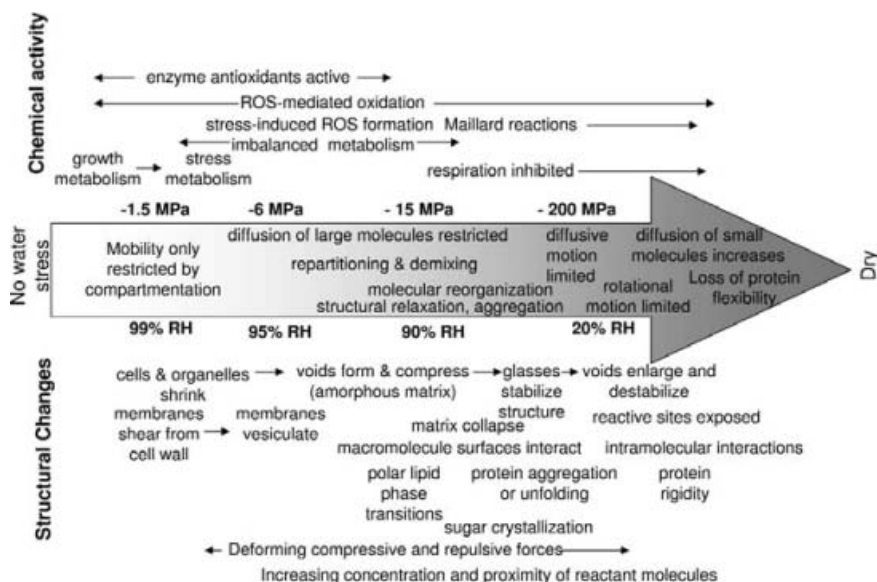


Fig. 9.3 Some direct (structural and chemical) and indirect (molecular mobility) effects of dehydration on cells. Levels of hydration are defined by the types of reactions that are induced by drying or by the degree to which mobility is restricted. The extent to which structural and chemical changes occur in desiccation tolerant and sensitive systems is dependent on the driving force of the reaction (e.g., changes in concentration or chemical potential), the type of mobility required for the particular reaction, and the degree to which mobility is restricted.

that current assessments of damage only detect irreparable changes. These changes are classified as physical derangement of structure or biochemical degradation of molecules. We believe that damaging reactions are induced at threshold water potentials (Ψ_{n_x} where the subscript x represents a particular type of damaging reaction). The extent to which these reactions occur is a function of the difference between the threshold water potential and the water potential of the cell (Ψ) and barriers or catalysts to the reactions that result from the physical or physiological state of the cell. The function takes the familiar form of Darcy's law, where

$$J \cong \frac{\Psi_{n_x} - \Psi}{R} = \frac{\Delta\Psi}{R} \quad \text{Eq. 1}$$

J describes the kinetics of the damaging reaction and R is the sum of resisting and catalyzing factors. For example, the threshold water potential for phase transitions of membrane polar lipids Ψ_{membrane} is about -10 MPa (Wolfe 1987, Bryant and Wolfe 1989, Crowe et al. 1989, 1990, Koster et al. 1994, 2000, Stepunkus et al. 1995, Hoekstra and Golovina 1999, Wolfe and Bryant

1999). Hence, drying cells to $\Psi_w \leq -10$ MPa may allow a fluid-to-gel phase change in polar lipids to occur; the relative rate of this damaging reaction is dependent on the fatty acid composition (e.g., saturated fatty acids tend to crystallize more readily than unsaturated fatty acids) and the presence of protecting solutes that separate interacting polar lipids. Water controls the physical and biochemical status of a multitude of cellular constituents and we hypothesize that each has a threshold water potential and ameliorating factors. Fig. 9.3 describes potential damaging reactions and their hypothesized threshold water potentials.

The idea that there are threshold water contents or water potentials for damaging reactions (Fig. 9.3) and critical water contents or water potentials for survival (Fig. 9.1) leads to the obvious hypothesis of a cause-effect relationship. For example, the critical water potential of about -12 MPa that is observed in rapidly dried recalcitrant embryos (e.g., Pammenter et al. 1991, Wesley-Smith et al. 2001) perhaps suggests that membrane phase changes, which can occur at $\Psi < -10$ MPa, are the mechanism of damage. With a notable exception (Conner and Sowa 2003), there are few data to support this hypothesis or the converse, that tolerant organisms do not suffer membrane perturbations (but see Seewaldt et al. 1981, Priestley and de Kruijff 1982, Hoekstra et al. 1999). The value of comparing threshold and critical water potentials is the understanding of the types of physical and chemical damage that are possible in systems dried to different hydration levels. Thus, phase changes within membranes are a less likely mechanism of damage in immature embryos and vegetative cells, which succumb at water potentials between -1.5 and -5 MPa. As cells are dried to lower Ψ , the list of potentially damaging reactions gets longer and the rate at which these reactions occur potentially increases ($\Delta\Psi$ in Eq. 1 increases). These damaging reactions are considered direct effects of dehydration; however, the rate at which they occur can be modulated by indirect effects of dehydration on viscosity, as described later.

All cells are subject to the same damaging reactions and the reactions are induced at certain hydration levels (Fig. 9.3). This chapter introduces the idea that a critical distinction between desiccation-tolerant and -sensitive cells is the rate at which these inevitable damaging reactions occur. From this perspective, differences in critical water potentials (Fig. 9.1) measured among species, tissue types, or drying protocols may be less of a reflection of differences in tolerance to the *intensity* of stress and more a function of differences in tolerance to the *duration* of stress. For example, a range of critical water potentials between about -10 and -30 MPa have been reported for mature, rapidly dried recalcitrant embryos (Pammenter et al. 1991, Vertucci and Farrant 1995, Pammenter and Berjak 1999, Sun and Liang 2001, Walters et al. 2002) and has led to the suggestion that more water can be removed with impunity in some species. The alternative interpretation suggested here is that

the same damaging reaction occurs at the same level of stress in each species, but there is a difference in how rapidly the reaction occurs. Hence, cells surviving to -30 MPa appear more tolerant than cells that survive to only -10 MPa because the damaging reaction in the more tolerant cells is slower. Drying to -30 MPa speeds up the damaging reaction by increasing its likelihood ($\Delta\Psi$ gets larger), but holding the cells longer at -10 MPa may have the same lethal effect. Extending the argument, the same damaging reaction may occur in highly tolerant cells, but the time scale in which the reaction occurs is impractical to measure experimentally. Thus, variation in the parameter R in Equation 1, which describes factors that increase (e.g., catalysts) or decrease (e.g., inhibitors) reaction rate, can profoundly define relative desiccation tolerance among cells. Fig. 9.3 lists several mechanical, biochemical, and biophysical perturbations that occur when water is removed from cells. Building from this list and our working hypothesis, we can now view the lethal mechanism of desiccation as the fastest reaction given the particular circumstances of the cell and the drying and storage protocol.

The role of kinetics in desiccation damage originates from colleagues Patricia Berjak and Norman Pammenter, who demonstrated that survival of recalcitrant seeds was higher if the tissues were dried faster (Berjak et al. 1989, Pammenter et al. 1991, 1994, 1998). At the same time, Wesley-Smith (1992, 2001b, 2004) demonstrated that recalcitrant seeds survived liquid nitrogen temperatures when cooled rapidly. These scientists showed that many of the damaging reactions of drying and cooling could be avoided if cells were processed faster than lethal reactions occurred. We also demonstrated the converse—that desiccation sensitivity could be induced in normally tolerant tissues by simply prolonging desiccation treatments (Walters et al. 2001). The assays used to induce desiccation damage in otherwise tolerant tissues are the high humidity conditions known as “controlled deterioration” or “accelerated aging” in the seed quality literature (Delouche and Baskin 1973). The link between desiccation damage and aging is also implied by the “ultradry” controversy over optimum storage conditions for seeds (Walters 1998a). Seeds and other anhydrous organisms sometimes deteriorate more rapidly when stored at water potentials less than about -200 MPa (22% RH at 25°C) (Walters et al. 2005).

The idea that the quantitative nature of desiccation tolerance is manifested in the time that it takes for damaging reactions to occur has some important implications for experiments that compare critical water potentials among cells or that correlate presence of cellular constituents with survival. Future experiments might well distinguish degrees of desiccation tolerance among organisms by their survival times at a single stressful water potential rather than measuring critical Ψ (Fig. 9.1). Measuring failure rate at a constant water stress would lead to more specific assays of damage and protection. This experimental approach was used previously to determine yield times during

osmotic stress and to develop useful kinetic models (Williams et al. 1993). Kinetic models of desiccation damage may also lead us to reevaluate experiments that correlate concentration of cellular constituents believed to cause (e.g., ROS) or protect against (e.g., sugars) desiccation damage. The effect of concentration on kinetics is hardly defined for desiccation-induced reactions, and reactant or inhibitor concentration may have different, even negligible, effects for zero to multiordered or cooperative reactions. Further, the effect of cellular constituents on R in Equation 1 is likely to have a complex relationship with concentration.

9.4 Moisture “Rafts”

This chapter describes structural and biochemical changes that occur as cells dry. Drying (or rehydration) need not be a homogeneous process, and there are reports suggesting that local pockets of water remain as organisms dry or are created upon rehydration. Presumably, these pockets of higher water availability or mobility are different than high water holding capacity (e.g., Fig. 9.2, water absorption varies among tissue types), and represent a relatively unexplored mechanism to protect cells from water stress by avoidance or to stimulate local metabolism.

Two hypotheses can be suggested to explain what appears to be the preferential collection of water. Differences in water diffusivity among cells and tissues may create barriers or conduits to moisture flow (R in Eq. 1) resulting in ephemeral pools of high and low water potentials. The literature on diffusion properties among seed parts and water movement through grains is rich and supports this notion (e.g., Vertucci et al. 1989, Allen et al. 2000, Casada 2002, Manz et al. 2005, Cloetens et al. 2006, Kikuchi et al. 2006).

An alternative hypothesis for water pockets is that regions of varying molecular mobility, albeit at the same water potential, coexist (e.g., Bruni and Leopold 1992, Balsamo et al. 2005, implied by Leubner-Metzger 2005). This mechanism would require localized concentrations of solutes that affect mobility, but not water status, within cells. In other words, cells dry, but the indirect effects of dehydration on viscosity, as discussed later in the chapter, would be spatially distributed and vary from cell to cell or from organelle to organelle depending on solutes. Candidate molecules would be sugars and LEA (late embryogenic abundant) proteins, which are highly regulated in response to water stress and have profound effects on the properties of aqueous solutions (Walters et al. 1997, Wolkers et al. 2001, Reyes et al. 2005). As we will see, mobility within drying systems is a double-edged sword that can initially mask the effects of dehydration but will eventually lead to undesirable reactivity or structural collapse with extreme drying. LEA proteins in

the presence of sugars have been hypothesized to serve as “hydration buffers” that provide transient protection during dehydration (Walters et al. 1997).

Apparent increases in the molecular mobility of water with drying is a historically compelling observation which is difficult to explain. The renowned “water replacement hypothesis” was initially supported by the observation of increased proton relaxation under very dry conditions, which was interpreted as a preferential binding of sugars to, and release of water from, macromolecule surfaces (e.g., Clegg 1986). Similar increases in proton mobility (Pikal et al. 1992) and ESR-probe rotation (Buitink et al. 2000a) upon drying to water contents near -150 MPa have also been reported and beg the question of whether it is possible to consolidate water molecules in a tissue and create moisture rafts or whether our probes of mobility are specific enough. One might imagine that these moisture rafts—if they are indeed highly concentrated or mobile water—would have important detrimental consequences to survival of dried germplasm below the water freezing temperature, provided that the pocket of water is sufficiently large to enable ice nucleation during freezing. We have not observed this situation (Walters 2004, Walters et al. 2005a, 2005b, 2007).

9.5 Reactions When Cells Dry

The many functions of water in cells—as a scaffolding and cement for cell structures, a fluid matrix for solute diffusion and intramolecular motion, a major factor determining pH and polarity, a powerful antioxidant, and an important substrate, product, or catalyst of many chemical reactions—imply numerous possible mechanisms for damage when water is removed. The effects of water removal are usually considered in terms of direct effects of dehydration on structure and chemistry (affecting $\Delta\Psi$ in Eq. 1) or indirect effects of viscosity changes (affecting R in Eq. 1). Attempts to isolate specific effects are important to understanding the role of putative protectants. However, the complexity of water interactions makes it difficult to identify single lesions and how they lead to cascades of structural (mechanical or biophysical) or biochemical changes that eventually cause irreparable damage. The following section provides a broad overview of reactions that occur as a direct consequence of dehydration. Although potentially damaging, it is not clear that these reactions actually occur within a relevant time frame or that they are lethal events.

9.5.1 Volume Changes in Cells

Mechanical perturbations resulting from cells shrinking and swelling during water loss and rehydration are among the first challenges of cell desiccation.

Cells lose turgor when water potentials fall below about -1 to -1.5 MPa and cell division and radicle protrusion are inhibited (Vertucci and Farrant 1995). Damage from plasmolysis is most evident at plasmodesmata where shearing of the plasma membrane is observed, and the resulting interruption of cell-cell communications likely interferes with cell development and recovery (Iljin 1957, Bewley and Krochko 1982, Murai and Yoshida 1998b, Farrant 2000, Vander Willigen et al. 2003). Drought-tolerant cells resist these mechanical stresses by accumulating compatible solutes and decreasing the cell osmotic potential (Levitt 1980, Wyn Jones and Gorham 1983, Farrant 2000). The high concentrations of solutes allow the cells to retain water and maintain cell volume. This strategy is effective at water potentials between -1 and -2.5 MPa (Wolfe and Bryant 1999). Cells cannot use osmotic adjustments for protection against more severe desiccation stress ($\Psi < -3$ MPa).

Mechanical perturbations from cell shrinkage are inevitable under more severe desiccation stress (< -2.5 MPa). In the 1970s, Meryman introduced the concept of "minimum critical volume" in which he suggested that an approximately 50% reduction in cell volume was lethal (Meryman 1974). This idea was modified by Steponkus and colleagues, who suggested that loss of cell surface area was critical (Steponkus and Lynch 1989, Steponkus et al. 1995). Steponkus's group showed that the plasma membrane of mesophyll protoplasts folded outward or inward during shrinkage. Extracellular folding was correlated with cell survival despite a greater than 50% reduction in cell volume, while intracellular folding appeared lethal because membrane material vesiculated and separated from the plasmalemma. In Steponkus's model, the initial loss of cell volume or plasmalemma surface area is not damaging per se. The damage occurs when the shrunken cell is returned to the original water potential and subsequently bursts because there is insufficient membrane surface area to accommodate the swelling. Vacuoles within plant cells are also susceptible to shrinking and swelling perturbations, which may explain greater tolerance in undifferentiated or less vacuolated cells (Farrant et al. 1997, Murai and Yoshida 1998a, Farrant 2000, Vander Willigen et al. 2003).

Mechanical perturbations from osmotic shrinkage and swelling usually occur at threshold water potentials between -2 and -5 MPa. Meryman and Steponkus demonstrated this empirically, but similar conclusions can be intuited from sorption isotherms (Fig. 9.2) which show a steep relationship between water content and water potential at $\Psi_w > -5$ MPa. The water that is present in cells at high water potentials occupies volume, which is lost when the cells dry. The extent that cell volume changes during desiccation is directly related to the proportion of dry matter reserves that have been loaded into the cells (Fig. 9.4). Cells with low dry matter content are likely to be highly vacuolated and will shrink more than cells that are stuffed with insoluble dry matter reserves. For example, about 85% of the volume of a moderately

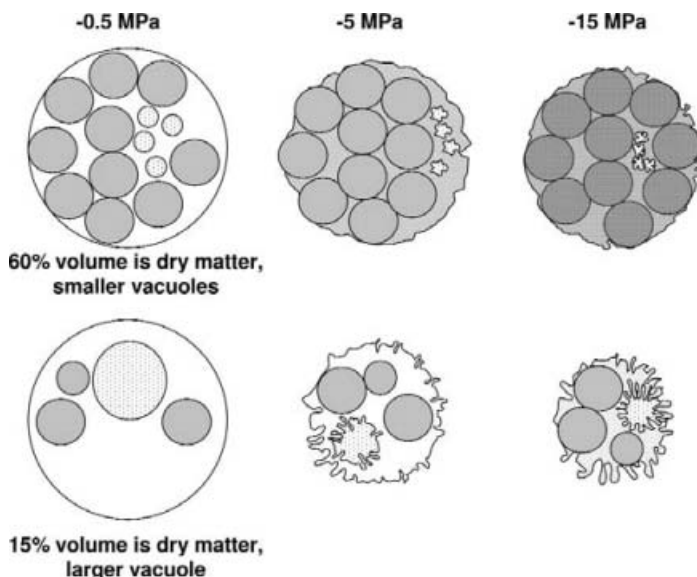


Fig. 9.4 Scaled drawing of osmotic shrinkage in cells containing different quantities of dry matter reserves. Diagrams are representative of the mature cotyledon and immature embryo described in Fig. 9.2. Cell volume was calculated assuming a 1:1 loss of mass to volume as water is removed. Changes in vacuole (*light-colored circles with dots*) volume were calculated assuming that 10% of the original volume was comprised of solutes. For color detail, please see color plate section.

vacuolated cell is occupied by water under isotonic conditions at -0.5 MPa. An isotherm of this cell shows that it will lose about $5 \text{ g H}_2\text{O} \cdot \text{g}^{-1} \text{ dw}$ when exposed to -5 MPa (Fig. 9.2) and this will cause a 75% reduction in volume (Fig. 9.4). In contrast, a cell from a mature pea cotyledon is loaded with protein bodies and starch grains and water occupies only about 40% of its volume at -0.5 MPa (Fig. 9.2). This cell will lose only $0.37 \text{ g H}_2\text{O} \cdot \text{g}^{-1} \text{ dw}$ when exposed to -5 MPa and the cell volume will decrease by less than 22% (Fig. 9.4); the cell volume will decrease by less than 40% upon complete drying. Using Meryman's critical volume change of $>50\%$, cells containing 50% or more dry matter under isotonic conditions will completely avoid mechanical damage from osmotic cell shrinkage and swelling. Cells containing less than 25% dry matter will be damaged when water potentials are reduced below -2 MPa. These highly susceptible cells may benefit from osmotic adjustments.

Cells with more dry matter reserves are more tolerant of desiccation because they have greater ability to resist mechanical perturbations during water removal (Vertucci and Farrant 1995, Farrant et al. 1997). The dry matter accumulation phase of embryogenesis is therefore a critical step toward greater

desiccation tolerance in seeds, and reserve mobilization directly leads to loss of desiccation tolerance. During reserve accumulation, the volume of dry matter within embryonic cells increases from about 10% at posthistodifferentiation to 40% or more before the vascular connection between embryo and mother plant is severed. Water, which originally gave organelles structure and rigidity, is displaced by storage proteins, starch, and triacylglycerols. Critical water contents defining desiccation damage decrease from about 1.2 to about $0.3 \text{ g H}_2\text{O} \cdot \text{g}^{-1} \text{ dw}$ during the dry matter accumulation phase of embryogenesis (Berjak et al. 1992, Finch-Savage 1992, Sun and Leopold 1993, Vertucci and Farrant 1995, Farrant and Walters 1998). Despite this dramatic change in critical water content, the critical water potential remains between -3 and -5 MPa (Farrant and Walters 1998, Walters 1999), resulting in changes of more than 50%, as predicted by Meryman. Therefore, accumulation of dry matter increases a cell's tolerance for water removal but does not necessarily affect the minimum water potential that a cell tolerates. Mechanisms that facilitate displacement of water by dry matter, by definition, enhance tolerance for water removal and possibly explain the correlation between catalysts for water movement, such as aquaporins, and desiccation tolerance (e.g., Smith-Espinoza et al. 2003).

9.5.2 *Collapse of Matrix and Molecule Structure*

Cells and their contents shrink during dehydration. Initially, the ratio of water loss to volume loss is 1:1 (assuming density of water is 1 g/ml). However, as dehydration proceeds that ratio increases as voids form in the aqueous cellular matrix (Fig. 9.5). These voids or pores are a consequence of imperfect packing of molecules as they condense and compress. Strong intramolecular hydrogen bonding helps to preserve the folded structure of most proteins and other large molecules when water is removed from the interior and surface of molecules. Intermolecular hydrogen bonds form among solutes and macromolecular surfaces, creating an amorphous, porous matrix. As drying progresses, hydrogen bonding increases and molecular mobility decreases. With extreme drying, amorphous structures become "frozen-in" because the high viscosity precludes further structural changes within the measured time scale. The cellular matrix vitrifies at water contents below about $0.1 \text{ g H}_2\text{O} \cdot \text{g}^{-1} \text{ dry mass}$ at room temperature ($\Psi_w \approx -100 \text{ MPa}$ and $\text{RH} \approx 50\%$) in a state change called a glass transition (T_g). The actual water content at the glass transition is an inverse function of the temperature and the time scale used to measure structural changes, as well as the hydrophilic properties and molecular mass of the glass former. Differences in desiccation tolerance and longevity among seed species were once hypothesized to result from differences in T_g (Koster 1991, Leopold et al. 1994, Buitink and Leprince 2004). However,

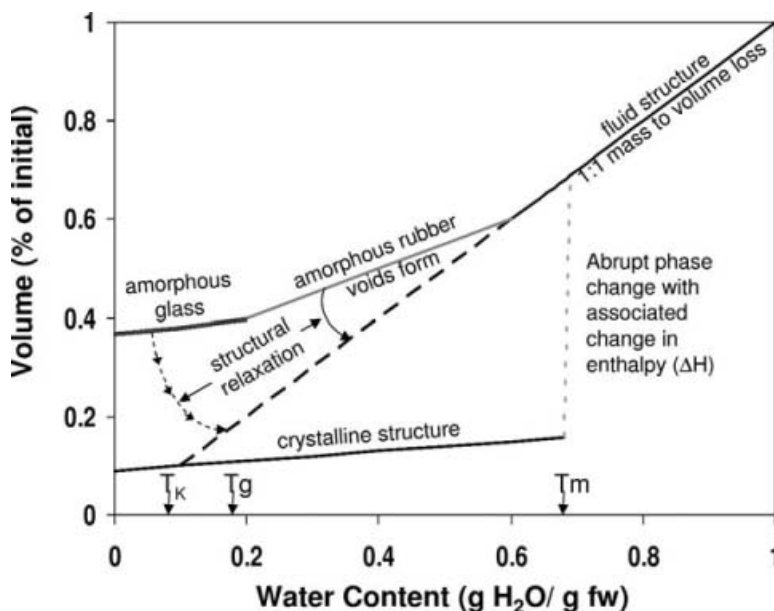


Fig. 9.5 A model describing the effect of water loss on the volume of an aqueous solution such as cytoplasm. The model shows the initial 1:1 mass-to-volume loss expected for osmotic shrinkage (Fig. 9.4). Depending on temperature, drying rate, purity, and molecular complexity, a molecule may bond with like molecules and crystallize out, causing an abrupt decrease in volume and molecular mobility at T_m . Alternatively, different solutes may hydrogen bond into an amorphous matrix that forms voids as a result of water removal and imperfect packing. All amorphous matrices “relax” to fill the voids and volume changes eventually tend toward the 1:1 volume:mass line (*dashed line*). Cataclysmic relaxation may occur at higher water contents in a structural failure known as “collapse” (*solid curved arrow*). Mobility progressively decreases with drying, until at some water content and temperature (T_g), motion is so restricted that the existing structure becomes “frozen in” and only small relaxations can be noted within a relevant time frame (*dashed curved arrow*). At the Kauzmann temperature (T_K), the mobility and volume of the amorphous matrix are similar (although structure is different), and so relaxation is essentially stopped though other types of molecular motion may still be allowed. This model is based on concepts of configuration entropy that are described in more detail for seeds in Walters (2004).

values for T_g appear similar among seeds with different storage characteristics (Buitink and Leprince 2004), suggesting that glass formation per se is not a feature that differentiates levels of desiccation tolerance.

The formation of intermolecular pores or voids and the resulting amorphous structure in the aqueous matrix can be beneficial or deleterious to dehydrating cells. In the short term, macromolecular structures and substrate availability are conserved. Over time, however, the physical integrity and chemical activity of amorphous structures become destabilized as the structures “relax” and compress pore space (Fig. 9.5). The hydration level marking mass:volume losses greater than 1 (i.e., voids are formed) has not really been explored for

biological materials but is estimated to occur at water potentials near -8 MPa according to water sorption characteristics (Zhang and Zografi 2000, Ballesteros and Walters 2007) and anomalous thermal properties of water (Vertucci 1990). The porous structure stabilizes when the matrix vitrifies at T_g , which occurs at water potentials between -50 and -150 MPa, depending on composition and temperature. Between water potentials/water contents marking pore formation and glass transition (approximately -8 to -150 MPa), the amorphous aqueous matrix is said to be in a rubbery state and is prone to collapse under its own weight (Fig. 9.3) (White and Cakebread 1966, Tsourouflis et al. 1976, Aguilera et al. 1993) (Fig. 9.5). Density increases when amorphous matrices collapse and pore structure is lost. The denser material becomes a barrier to diffusion of volatile molecules that once moved through the pores. Collapse often leads to crystallization of highly concentrated, small-molecular-weight solutes or irreversible changes of internal conformation in proteins (e.g., Singhal and Curatolo 2004, Thomas et al. 2004). In the food and pharmaceutical literature, collapse is catastrophic, resulting in a permanent loss of quality, functionality, and ease of processing. Collapse of amorphous structure has not been investigated as a mechanism of damage in dehydrating seeds, pollen, or spores or in tissues stored under warm, high humidity conditions, although the time, temperature, and moisture content dependency of the two phenomena are remarkably similar to conditions studied in the food and pharmaceutical sciences.

The kinetics of structural changes in amorphous aqueous matrices is a function of the water content, temperature and molecular properties of the solutes comprising the matrix. The rate of collapse within rubbery materials is related to the difference between the holding conditions (temperature and water content) and glass transition conditions ($T - T_g > 0$) (e.g., Karmas et al. 1992, Aguilera et al. 1993, Bell et al. 1998, Ludescher et al. 2001). In model systems, larger solutes appear to confer greater stability to amorphous structures before they have vitrified (Sastry and Agmon 1997, Bell et al. 1998, Allison et al. 2000, Passot et al. 2005). The risk of collapse decreases as conditions approach the glassy state because molecular mobility is increasingly restricted. A vitrified system is considered "metastable," meaning that the matrix is functionally stable within a relevant time scale. Eventually, however, a glassy matrix "relaxes," and its volume compresses over a prolonged period. Much of the material science literature recognizes the inevitability of structural changes within glasses and uses a practical time scale to measure stability—for example, a year or two when considering the shelflife of foods or pharmaceuticals (Shamblin et al. 1999, Singhal and Curatolo 2004, Thomas et al. 2004, Hill et al. 2005). The time-dependency of glass stability can be conceptually expressed by Equation 1, where J represents the rate of structural (i.e., volume) change for a particular level of dehydration that is driven by $\Delta\Psi$,

and R is a function of the molecular mobility. At high viscosity, R is large and J is small—but still positive. Thus, glasses never halt physical and biochemical activity, they only slow it down. The concept of amorphous matrix formation and relaxation is widely applicable to dried biological systems and provides a mechanism for desiccation damage that accounts for differences in moisture, temperature and time dependency in diverse plant structures.

9.5.3 *Phase Changes, Phase Separation, and Repartitioning*

Amorphous matrices described in the previous section result when cells are dried and intracellular constituents compress but do not undergo major structural changes. In other words, there are no abrupt volume changes with moisture loss and temperature fluctuations in amorphous matrices. Sometimes abrupt changes in molecular structure occur with changes in moisture or temperature and mark a phase transition. For illustration of the two types of structural changes, imagine drying a sugar solution. It becomes a sticky mess which solidifies into a candy lollipop with further drying (continuous volume change of amorphous materials), or it forms sugar crystals at the expense of the fluid (discontinuous volume change of a liquid to crystalline phase change) (Fig. 9.5). Crystallizations require intermolecular interactions between like molecules and sufficient time for molecules to restructure into a nonrandom matrix. Thus, molecular proximity, purity, and fluidity are essential factors allowing crystallizations to proceed. Crystallization reactions of sugars and lipids have been reported in dry foods and model systems (e.g., White and Cakebread 1966, Caffrey et al. 1988, Crowe et al. 1989, 1990, Koster et al. 1994, 1996, Hoekstra et al. 1999, Wolfe and Bryant 1999, Bryant et al. 2001, Walters et al. 2002, Thomas et al. 2004).

Sugar crystallization during drying has mostly been observed in foods or simple model systems with high sugar contents (e.g., White and Cakebread 1966, Leopold and Vertucci 1986, Caffrey et al. 1988, Koster et al. 1994, 1996, Costantino et al. 1998a, Thomas et al. 2004). Crystallization of sucrose following collapse of the rubbery amorphous mixture can be inhibited by adding impurities such as raffinose or proteins to the mixture to stabilize the amorphous structure. Translation of these observations to the desiccation-tolerance literature led to the hypothesis that sucrose protection of macromolecule structure was lost if it crystallized out of solution; hence it was proposed that oligosaccharides were required to limit sucrose–sucrose interactions (Leopold and Vertucci 1986, Koster and Leopold 1988, Koster 1991, Leopold et al. 1994, Sun et al. 1994). An extension of the hypothesis led to the suggestion that longevity in dry seeds was promoted by accumulation of sucrose and oligosaccharides (e.g., Leopold et al. 1994, Horbowicz, and Obendorf 1994, but see Vertucci and Farrant 1995, Buitink et al. 2000, Walters et al.

2005). Sugar concentrations change during seed maturation and germination commensurate with these hypotheses; however, there is no evidence that sucrose crystallized in cells that did not survive drying or storage.

Observations that desiccation-damaged cells profusely leak cellular constituents when they are rehydrated led to the hypothesis that the membrane is the primary site of damage during desiccation (or rehydration) (Steponkus et al. 1995, Hoekstra et al. 1997, 1999, 2001, Halperin and Koster 2006, reviewed in Walters et al. 2002). Polar lipids undergo phase transitions at threshold water potentials (Ψ_{membrane}) near -10 MPa (Wolfe 1987, Bryant and Wolfe 1989, Crowe et al. 1989, 1990, Steponkus et al. 1995, Hoekstra et al. 1997, 1999, Wolfe and Bryant 1999, Koster et al. 2000)—comparable to critical water contents reported for rapidly dried recalcitrant seeds and pollens (Walters et al. 2002). Phase changes within polar lipids have been reported in dry seeds and pollens (Seewaldt et al. 1981, Priestley and de Kruijff 1982, Hoekstra et al. 1997, 1999, 2001), but these changes are subtle and largely reversible until cells die (Conner and Sowa 2003). Phase transitions of membranes arise from the compression of cell constituents during drying. As cells shrink, polar lipid lamellae are brought into close proximity (Fig. 9.4), creating large deforming hydration forces between appressed bilayers (Wolfe 1987, Wolfe and Bryant 1999, Bryant et al. 2001), and eventually allowing interaction of adjacent head groups once the structural water around them is removed (Crowe et al. 1989, 1990, Hoekstra et al. 1997, 1999, Buitink et al. 2002). There are several crystalline structures formed by lipids depending on temperature, water content, cooling and drying rate, length and degree of saturation of fatty acyl chains, and geometry of the head group (Small 1986). Lamellar structure is maintained in a liquid crystalline to gel transition, which is the most common phase change observed in membrane lipids. Hexagonal crystals, in which the head groups coalesce into rings and acyl chains extend radially outward, is the predicted structure when there are high concentrations of phosphatidylethanolamine diglycerols or monogalactosyl diglycerols, as are found in chloroplasts. This type of crystalline structure facilitates exchange between bilayers and consequently membrane fusion and loss of compartmentation (Steponkus et al. 1995). There are few reports that different endomembrane systems fuse in plant reproductive structures, perhaps because plastids and mitochondria dedifferentiate during maturation and concentrations of non-lamellar-forming polar lipids are therefore reduced (Vertucci and Farrant 1995, Walters et al. 2002).

Phase transitions within polar lipids of membranes are dependent upon temperature as well as water content. Hydrophilic and amphiphilic solutes that migrate near or into the membrane matrix can depress the phase transition temperature but do not inhibit the transition altogether (reviewed by Bryant et al. 2001, Hoekstra et al. 2001). Presumably, fluid-to-gel phase changes occur in membranes of orthodox seeds and pollens that survive cold storage

or cryopreservation; when imbibed properly, cold-treated germplasm is undamaged (Hoekstra et al. 1999). One may conclude, therefore, that fluid to gel phase changes in lipids are fully reversible and are not damaging per se. Difficulties arise when cells with rigid membranes are exposed to liquid water and transient damage may occur until the semipermeable and elastic properties of fluid membranes are reestablished to prevent loss of cellular constituents and to accommodate swelling (Steponkus et al. 1995, Hoekstra et al. 1999).

Close appression of membrane surfaces and phase transitions of the polar lipid fraction can lead to other structural changes that are potentially irreparable. Demixing of membrane constituents occurs as similar molecules aggregate to accommodate compressive forces (Bryant and Wolfe 1989, Wolfe and Bryant 1999). Repartitioning and phase separation leads to membrane domains enriched with particular lipid classes or proteins, but potentially lacking molecules that might inhibit further structural change or chemical activity (reviewed by Hoekstra et al. 2001, Walters et al. 2002). Amphiphilic molecules from the cytoplasm may partition into membrane surfaces as volume and polarity change upon desiccation and change the ordered packing of polar lipids (Golovina and Hoekstra 2002, 2003).

Though less studied, phase transitions of triacylglycerols also appear to be associated with damage in relatively dry seeds (Crane et al. 2003, 2006, Volk et al. 2006). As with phase transitions of polar lipids in membranes, crystallization of triacylglycerols does not appear to be damaging per se. However, slight hydration of cells with crystallized lipids resulted in complete and irreversible cellular disruption (Volk et al. 2006). The mechanism of damage is unclear; however, the critical water content for damage (Fig. 9.3) suggests hydration-dependent repartitioning of amphiphilic substances within the oil body and interaction of those substances with other cellular components. The critical hydration level ($\Psi_w \geq -40$ MPa) and the kinetics of triacylglycerol crystallization (Crane et al. 2006) are consistent with loss of viability in seeds with so-called intermediate storage physiology and may potentially explain the appearance of partial desiccation tolerance in seeds that accumulate medium- and long-chain saturated fatty acids.

9.5.4 *Molecular Motion and Structural Changes in Proteins in the Amorphous Matrix*

Structural changes of proteins in drying systems is an important aspect of the foods and pharmaceutical industry, and we draw on the literature in those fields to gain insight on protein stability as plant reproductive structures are dried. Proteins vary in their stability to desiccation. Some enzymes, such as lactate dehydrogenase and phosphofructokinase, and polypeptides, such as poly(L)lysine, are highly labile during desiccation and require protection,

which can be afforded by sugars (Arakawa et al. 1993, Franks 1994, Sastry and Agmon 1997, Allison et al. 1999, Hill et al. 2005, Tang and Pikal 2005). For the most part, however, the native structure of proteins is initially maintained during drying, with only slight reversible changes resulting from compressive forces when water is removed (e.g., Griebenow and Klibanov 1995, Costanino et al. 1998). In seeds and pollen, changes in secondary structure (e.g., loss of α helical structure, increase in β folding) have been noted with lethal desiccation or aging (Golovina et al. 1997, Wolkers et al. 1998, Connor and Sowa 2003).

Structural changes to proteins in amorphous mixtures eventually occur and the kinetics of denaturation are dependent on the protein, moisture, temperature, time, and the presence of smaller-molecular-weight solutes (Sastry and Agmon 1997, Allison et al. 1999, Breen et al. 2001, Chang et al. 2005, Hill et al. 2005, Tang and Pikal 2005, Shamblin et al. 2006). Viscosity (a parameter of R in Equation 1) plays a critical role in the stability of proteins and foods at low water content, and our concept of viscous motion has dramatically changed over the last two decades. Early applications of the role of viscosity in molecular stability demonstrated that proteins were unstable above T_g , and the rate of structural collapse or aggregation was roughly predicted by the difference between the holding temperature and T_g ($T - T_g$) (Tsouroflis et al. 1976, Karmas et al. 1992, Aguilera et al. 1993, Franks 1994). Viscosity was assumed to be near infinity below T_g , giving undue optimism that structures would be forever stabilized once a glass formed. However, numerous studies have demonstrated an uncoupling between T_g and protein stability or have shown that sugars have anomalous effects on mobility (Sastry and Agmon 1997, Breed et al. 2001, Chang et al. 2005, Tang and Pikal 2005, Shamblin et al. 2006). Viscosity is now viewed as a complex function of translational, rotational, and vibrational motion, and T_g is only one measure of translational mobility over a narrow range of temperatures (Angell et al. 1994, Shamblin et al. 2006). The rates of protein unfolding and aggregation tend to correlate with structural relaxation within glassy matrices, or $T - T_g$ for T close to T_g , because these structural changes require diffusive motion. In other proteins, loss of activity may result from localized rotations or vibrations that are not coupled with structural relaxation, and methods to quantify other forms of viscosity are required (Sastry and Agmon 1997, Breed et al. 2001, Tang and Pikal 2005, Shamblin et al. 2006). Thus different types of motion influence the flexibility and reactivity of proteins and the dependency of these motions on water content and temperature varies among materials. Similar conclusions can be drawn from analogous experiments using seeds, which also show a decoupling between T_g and stability (Buitink et al. 2000a, Walters 2004, Walters et al. 2005a). The richness of correlation between stability and viscosity may call for multiple levels of solute-mediated control of mobility within the heterogeneous amorphous matrix of a cell, and may explain the complex

array of aqueous solutes, such as LEA and heat shock proteins, that are produced in desiccation-tolerant systems (Boudet et al. 2006, Stupnikova 2006, Macherel 2007).

9.5.5 *Chemical Reactions*

Changes in molecular proximity, concentration, structure, and mobility during desiccation have profound effects on the nature and kinetics of chemical reactions. Changes in metabolism are noted during the initial stages of desiccation stress at water potentials near -2 MPa (Black and Pritchard 2002, Illing et al. 2005, Buitink et al. 2006). These early responses to water stress are likely to confer greater tolerance within cells; however, their high metabolic cost may adversely affect the energy balance within the cell or lead to poor regulation of other housekeeping needs if the desiccation stress goes unrelieved (Fig. 9.3).

Metabolism appears to be detrimental to cells held at water potentials below about -3 to -5 MPa (Fig. 9.3). The observation that seeds with high respiratory activity succumb faster under water-stressed conditions suggests that metabolism is pathological or imbalanced (Leprince et al. 1996, 1999, 2000, Walters et al. 2001). Considering that hydration, temperature, and solutes affect proteins and polar lipids structures differently, it is conceivable that reaction sequences become uncoordinated under water-stressed conditions and toxic intermediates accumulate at the expense of needed products of metabolism. A simple example of uncoupling of the reaction $A \rightarrow B \rightarrow C$ with temperature can be used to illustrate (Franks 1985). Let us assume that under ideal conditions, the rate constants for $A \rightarrow B$ and $B \rightarrow C$ are arbitrarily assigned as 0.05 and 0.1 minute^{-1} , such that the concentration of B rises only slightly initially and then declines to a low steady state as the concentration of C rises. Dr. Franks shows us that if the reactions $A \rightarrow B$ and $B \rightarrow C$ have different temperature coefficients (e.g., activation energies) and, for example, decrease by 50% and 70% , respectively, for each 10° drop in temperature, B would accumulate at the expense of C . By analogy, a 50% and 70% change in reaction rates for $A \rightarrow B$ and $B \rightarrow C$ for each 1 -MPa decrease in water potential would result in accumulation of B . If the intermediate B were toxic, the effects of water stress would be devastating. In seeds, the slower diffusion of oxygen at about -6 MPa (Leprince and Hoekstra 1998), would result in a relatively high moisture coefficient for oxidative phosphorylation. This, in turn, may explain the increase in respiration and the accumulation of ethanol, acetaldehyde, and ROS in desiccation-damaged tissues (e.g., Akimoto et al. 2004, Calucci et al. 2004, Pukacka and Ratajczak 2005).

Oxidative reactions, mediated by ROS or the free radicals that readily form from them, degrade lipids, proteins, and nucleic acids. Damage occurs by the

abstraction of an electron from electron dense regions of molecules and perpetuates in auto-oxidation cascades that create more free radicals as well as small, reactive carbonyl byproducts that damage DNA and cause cross-links in proteins (Halliwell and Gutteridge 1999). Because they are electron dense, fatty acids in lipids are particularly susceptible to free radical attack. Oxygenated fatty acids and loss of double bonds cause increased membrane leakiness and decreased fluidity (Hoekstra et al. 2005).

Maillard reactions are another series of degradative reactions that have been implicated in desiccation damage (Wettlaufer and Leopold 1991, Sun and Leopold 1995, Murthy et al. 2003). These reactions are initiated by bonding between an aldehyde (usually a reducing sugar) and an amine group (usually the N-terminal of a protein). A reactive carbonyl intermediate, called an Amadori product, is further oxidized into an amine that perpetuates the Maillard reaction and smaller reactive carbonyl molecules that attack and oxidize proteins and DNA in reactions similar to those mediated by ROS. In foods, Maillard reactions are favored at relative humidity between 40% and 70% because there is sufficient water to allow the initial hydrolytic reaction, reactants are placed in close proximity, and the rubbery state allows molecular mobility (Karmas et al. 1992, Aguilera et al. 1993, Bell et al. 1998). Cells containing high concentrations of glucose and fructose are most susceptible to Maillard reactions, which may account for the high desiccation sensitivity of immature and germinating seeds (Koster and Leopold 1988, Vertucci and Farrant 1995, Black and Pritchard 2002) and for correlations between the accumulation of sucrose and other nonreducing oligosaccharides and desiccation tolerance.

9.5.6 *Chemical Versus Structural Change: What Comes First?*

Chemical reactivity requires some level of molecular mobility (Eq. 1), so the chemical and physical stability of amorphous matrices are inextricably linked. Physical order, such as within crystalline matrices, provides chemical stability because it allows less molecular mobility (Pikal and Dellerman 1989, Shamblin et al. 2006). However, abrupt changes in physical order may be detrimental. Conversely, disordered or amorphous systems move, albeit slowly once glasses form, and this motion promotes chemical reactivity. Hence, formation of amorphous structures is a double-edged sword. The challenge over the past two decades has been to quantify molecular mobility in complex systems and relate it to observed chemical degradation. In some cases, chemical degradation occurs faster than predicted by glassy relaxation. These reactions may still be diffusion driven but may involve small molecules that are not “frozen” into the glassy matrix and can move through its pores (Angell et al. 1994, Roozen et al. 1991). Alternatively, some chemical reactions require the

rotation of a functional group, and this type of mobility is not described by structural relaxation rates. Different types of molecular motions are involved in different chemical processes and are differentially affected by water. Perhaps this explains the existence of hydration levels for physiological activity, damage, and water properties in seeds (Fig. 9.3; Vertucci 1990, Vertucci and Farrant 1995, Walters 1998). Understanding of the mechanisms of these reactions and how the enabling molecular mobility is regulated will be the topic of future research.

9.5.7 *Ultralow Water Contents*

The principles of chemical reactivity and structural stability described in this chapter apply even at extremely low water contents. Hence, we expect complex interactions between water content, temperature, and different types of molecular mobility. Numerous studies referenced above show that the flexibility of protein and lipid molecules is requisite for maintaining structure and function. As water is progressively removed from these molecules, flexibility becomes permanently impaired (reviewed in Wang 1999, Walters et al. 2002, Hill et al. 2005, Shamblin et al. 2006; also Hsu et al. 1992). Loss of activity may arise from a multitude of mechanisms that include refolding of chains to increase packing efficiency (Kuntz and Kauzmann 1974, Lusher-Mattli and Ruegg 1982, Breen et al. 2001), exposing charged sites on heme groups to promote free radical formation (Sanches et al. 1986, Labrude et al. 1987), removing internal water molecules needed for local motion (Doster et al. 1986, Sastry and Agmon 1997), increasing reactivity within void spaces (Roozen et al. 1991, Angell et al. 1994, Hsu et al. 1995), and increasing reactant concentration (Bell et al. 1998). Principles learned from the food and pharmaceutical literature can be used to explain decreased survival of organisms under extremely dry conditions (Walters 1998, Walters et al. 2005b).

9.6 **Conclusions**

Desiccating organisms invariably undergo large structural and chemical changes as they come to equilibrium with a dry environment. Desiccation also has the collateral effect of reducing molecular mobility. These direct and indirect effects of dehydration on cell integrity and function have opposite effects and interact in a suite of time-dependent reactions that define whether an organism survives (desiccation tolerant) or perishes (desiccation sensitive). We suggest here that the rate that damaging reactions occur is a distinguishing feature of desiccation tolerance and sensitivity.

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Section 3
Applications of Desiccation
Tolerance Research

10 *XvSap1*, a Desiccation Tolerance Associated Gene with Potential for Crop Improvement

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Climate variability in southern Africa poses a direct danger to food security in the region (Tschirley et al. 2004, Lovett et al. 2005). In general, most crop plants are highly sensitive to even a mild dehydration stress. Many stress-inducible genes have been identified over the past decade, and their functional roles in stress tolerance have been elucidated. The improvement of crop stress tolerance by targeting stress-related genes for genetic manipulation is therefore now feasible. Resurrection plants, such as *Xerophyta viscosa*, can tolerate extreme water loss (greater than 90%), which makes them ideal systems to study desiccation stress tolerance (Farrant 2000, Mundree et al. 2002). Genes isolated from such plants can be used to improve drought tolerance of essential food security crops. This will greatly benefit the agricultural sector globally. This would also provide developing countries within sub-Saharan Africa with the potential to produce crops that would be able to withstand the harsh environmental conditions and possibly provide greater yields.

To isolate novel genes involved in desiccation tolerance from *X. viscosa*, various gene mining strategies were used. These included strategies such as complementation by functional sufficiency and differential screening of cDNA libraries. These approaches were successful in isolating a number of genes upregulated in response to various stresses such as low temperature, high temperature, dehydration, and oxidative stresses (Mundree et al. 2006). Using the strategy of complementation by functional sufficiency, Mundree et al. (2002) isolated a cDNA, designated *XvSap1*, from a cDNA library derived from dehydrated *X. viscosa* leaves. An *Escherichia coli* (srl::Tn10) mutant strain lacking the ability to take up and catabolize sorbitol was used to confirm whether *XvSap1* expression conferred osmoprotection. The *E. coli* mutant strain lacked the ability to take up and catabolize sorbitol. It was therefore unable to grow in minimal media, wherein sorbitol was the sole carbon and energy source as this osmoticum placed an osmotic stress on the cells. When *XvSap1* was expressed in *E. coli* (srl::Tn10) cells cultured in a high concentration of sorbitol, it was able to rescue the bacterium from the osmotic stress, confirming that *XvSap1* is associated with osmotic stress tolerance.

Sequence analysis of *XvSap1* predicted a highly hydrophobic protein with six transmembrane regions and two prokaryotic membrane lipid attachment sites (Garwe et al. 2003). These features suggest that *XvSap1* is anchored within the plasma membrane. The deduced amino acid sequence displayed

49% identity to WCOR413 from wheat (renamed TacCOR413-PM; Breton et al. 2003). This family of stress-inducible proteins consists of two distinct groups based on their intracellular localization—COR413 plasma membrane (COR413-PM) proteins and COR413 thylakoid (COR413-TM) proteins. The COR413 protein family has not been identified in other eukaryotic or prokaryotic databases, suggesting that it is unique to the plant kingdom (Breton et al. 2003).

Garwe et al. (2006) demonstrated that *XvSap1* expression in transgenic *A. thaliana* resulted in increased tolerance to salinity, high temperature, and osmotic stresses. The *XvSap1* gene was transformed into *A. thaliana* by Ti plasmid-mediated transformation under the control of the cauliflower mosaic virus 35S promoter. Transgenic *A. thaliana* expressing *XvSap1* grown in tissue culture maintained higher growth rates during osmotic, high salinity, and high temperature stresses.

To establish whether *XvSap1* was involved in abiotic stress at the transcriptional level in *X. viscosa*, analysis of gene expression using semiquantitative reverse transcription–polymerase chain reaction (PCR) was performed (Garwe et al. 2003). These results indicated that *XvSap1* is induced by dehydration (51% and 44% relative water content [RWC]), salinity stress (100 mM), both low (4°C) and high temperature (42°C), and high light treatment (1500 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). No transcripts of *XvSap1* were evident at 4% RWC or during rehydration, and it only started to accumulate at least 48 hours after high temperature and light stress. Furthermore, the *Xvsap1* transcript was detected within 24 hours after treatment with low temperature and correlated well with results obtained with other COR413 genes. It is known that high temperature stress causes denaturation of proteins, cellular enzymes, and damage to membranes (Karim et al. 1999), whereas light stress results in the formation of reactive oxygen species (ROS) that causes, among other effects, lipid peroxidation (Smirnoff 1993). The observed *XvSap1* mRNA expression and accumulation patterns together with the predicted association of the protein with the plasma membrane led Garwe et al. (2003) to suggest that *XvSap1* is not involved in the initial abiotic stress response but may play a role in the protection and repair of membranes against damage by heat/cold and light by maintaining structural integrity.

Salt shock of *X. viscosa* resulted in the *XvSap1* transcript appearing within 24 hours and persisting for 7 days. High exogenous salt concentrations cause an imbalance of cellular ions resulting in ion toxicity, osmotic stress, and production of ROS (Hasegawa et al. 2000). Various proteins that protect membrane integrity, control ion homeostasis, and play a role in ROS scavenging have been reported to attenuate salt stress effects (Serrano and Rodriguez-Navarro 2001, Yamaguchi and Blumwald 2005). A region of *XvSap1* from 36 to 119 amino acids bears 12% identity with a potassium transporter family that is conserved across bacteria, yeasts, and plants (Quintero and Blatt 1997).

It was therefore also suggested by Garwe et al. (2003) that *XvSap1* may possibly be involved in transport of small molecules or ions across the plasma membrane.

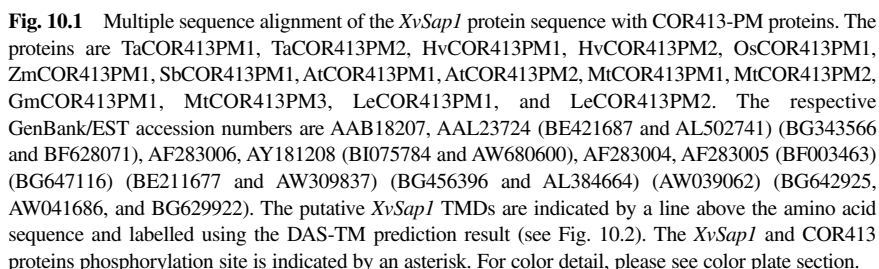
A combination of bioinformatics analyses and real-time quantitative expression profiling was used to obtain further insight into the function of *XvSap1*. This approach was previously used by Breton et al. (2003) to investigate the COR413 proteins from a variety of plant species. The *XvSap1* protein displays greater than 55% overall identity with the TaCOR413 and consequently clustered with the COR413 proteins. Analyses of the *XvSap1* protein sequence together with the other members of the COR413 family shows that there are regions of identity except for the first and last amino acids that are poorly conserved (Fig. 10.1). Residues such as proline and cysteine, which are important for protein structure and activity, are conserved in all the COR413-PM proteins and in *XvSap1*. The *XvSap1* protein has two α helices in the amino-terminal region similar to the COR413-PM proteins, but it does not appear to have a very G-rich region separating the helices compared with the other COR413-PM proteins (Fig. 10.1).

Hydropathy predictions on *XvSap1* using the DAS-TM program (Cserzo et al. 1997) indicated that the protein is highly hydrophobic (Fig. 10.2). In addition, transmembrane predictions on *XvSap1* using the TMHMM topology prediction program (Kyte and Doolittle 1982, Krogh et al. 2001) indicated that the protein has either six or seven transmembrane domains (TMD; Fig. 10.2). Based on these predictions, it is evident that *XvSap1* shares a very similar hydrophobicity and TMD profile to TaCOR413-PM (Fig. 10.3). In addition, prediction of the subcellular localisation of *XvSap1* using the WoLF PSORT analysis program (Horton et al. 2006) suggested that the plasma membrane was the most likely location for *XvSap1*.

The use of NetPhos phosphorylation prediction software (Blom et al. 1999) on the *XvSap1* protein sequence suggested only one phosphorylation site on the threonine residue. This predicted phosphorylation site on *XvSap1* is in a similar position to the previously predicted phosphorylation site for COR413-PM proteins (see Fig. 10.1; Breton et al. 2003). Glycosylated phosphatidylinositol (GPI) anchors target a protein to the cell surface (Chatterjee and Mayor 2001). However, unlike the COR413-PM proteins no GPI anchoring sites at the carboxyl -terminus were predicted for *XvSap1* using the DGPI program (Kronegg and Buloz 1999) and GPI Prediction server (Eisenhaber et al. 2003).

Given the similarity of *XvSap1* with COR413-PM proteins, phylogenetic analyses of *XvSap1* with members of this protein family was performed (Fig. 10.4). Interestingly, the phylogenetic analysis indicates that although *X. viscosa* is a monocotyledonous plant, the *XvSap1* protein is more closely related to the COR413-PM proteins from *A. thaliana* and *Lycopersicon esculentum* (Fig. 10.4).

Breton et al. (2003) proposed two structural models for COR413 proteins based on their TMD topology predictions. The first model proposed that the



COR413-PM proteins have five TMDs with the amino-terminal end outside and the carboxyl-terminal end inside. The second model proposed six TMDs with both amino-terminal and carboxyl-terminal ends within the cytoplasm. In both COR413-PM models, the sixth TMD was separated from the rest of the proteins due to the GPI anchor. These analyses and our membrane topology predictions allowed us to propose structural models for *XvSap1* (Fig. 10.5). Two models were proposed for *XvSap1*; in the first there are seven TMDs with

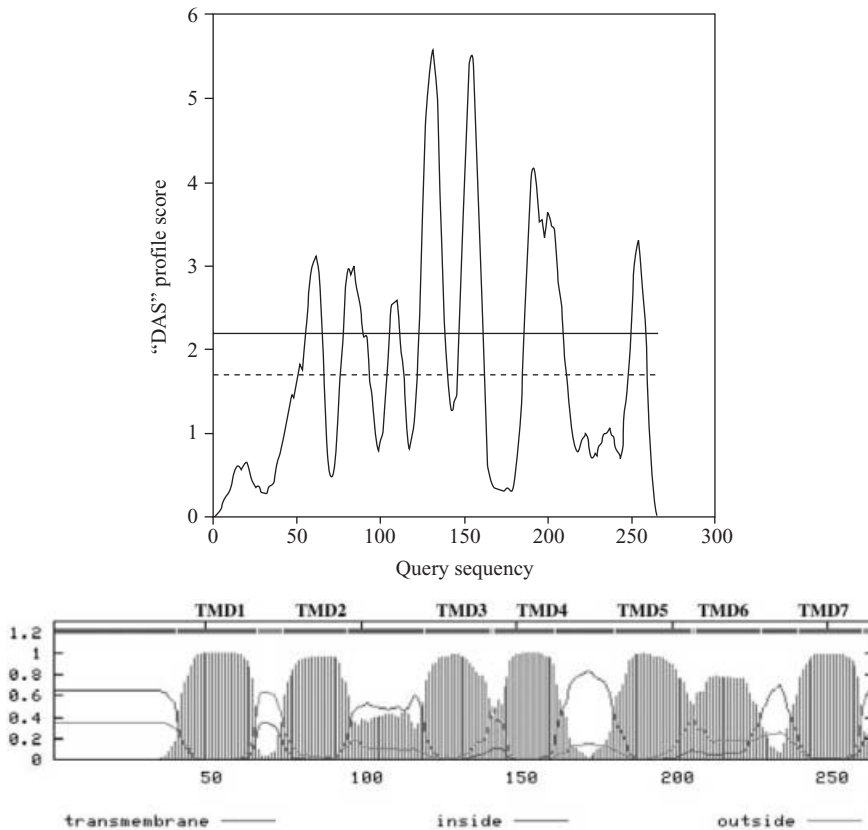


Fig. 10.2 Hydropathy and transmembrane predictions. (A) DAS-TM prediction. The *dashed line* represents the loose cutoff; *solid line* represents the strict cutoff. (B) TMHMM topology profile. The transmembrane helices are indicated above the profile. For color detail, please see color plate section.

the amino-terminal end inside and the carboxyl-terminal end outside (Fig. 10.5 Model 1). The second model has six TMDs with both terminal ends on the outside (Fig. 10.5 Model 2). In both *XvSap1* and COR413-PM proteins, the phosphorylation site occurs intracellularly (Fig. 10.5).

Breton et al. (2003) also demonstrated that COR413-PM genes were induced by the exogenous application of abscisic acid (ABA). Transcriptional analyses of *XvSap1* in response to the external application of ABA on *X. viscosa* plants using quantitative real-time PCR (qPCR) were performed. The qPCR analysis indicated that *XvSap1* mRNA was upregulated within 6 hours of *X. viscosa* being treated with ABA exogenously (Fig. 10.6A). This is in contrast to the findings of Garwe et al. (2003), which demonstrated no induction

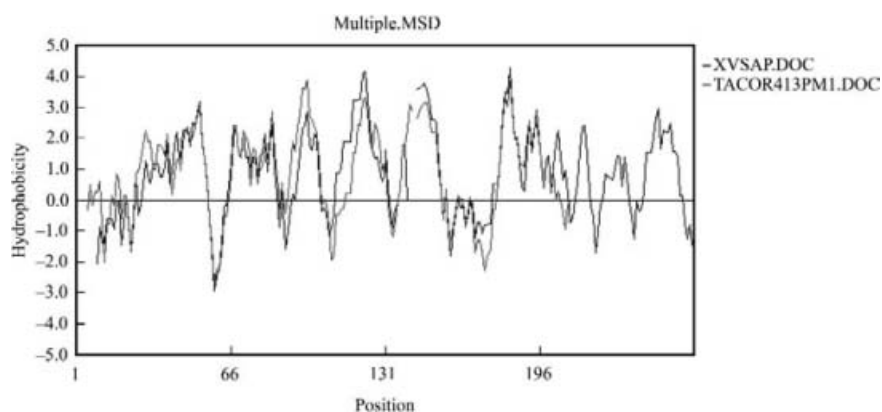


Fig. 10.3 Hydrophobicity overlap profile of *XvSap1* with TaCOR413PM protein as determined by the method of Kyte and Doolittle (1982). For color detail, please see color plate section.

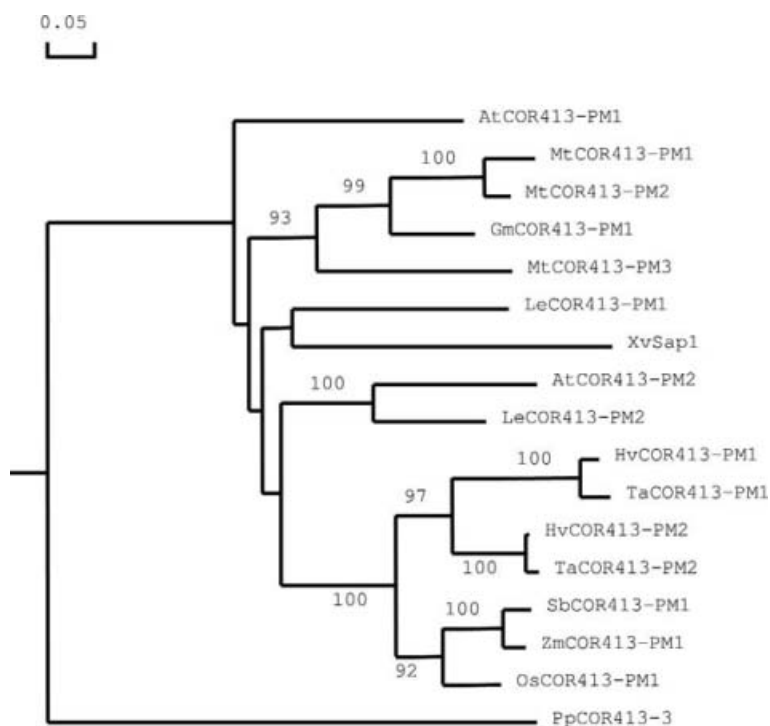


Fig. 10.4 Phylogenetic tree inferred from maximum likelihood analysis of *XvSap1* and COR413-PM protein sequence data. Numbers above the branches indicate bootstrap proportions (percentage of 100 replicates; values below 90 are omitted). The *bar* depicts 1 base substitution per 20 nucleotide sites.

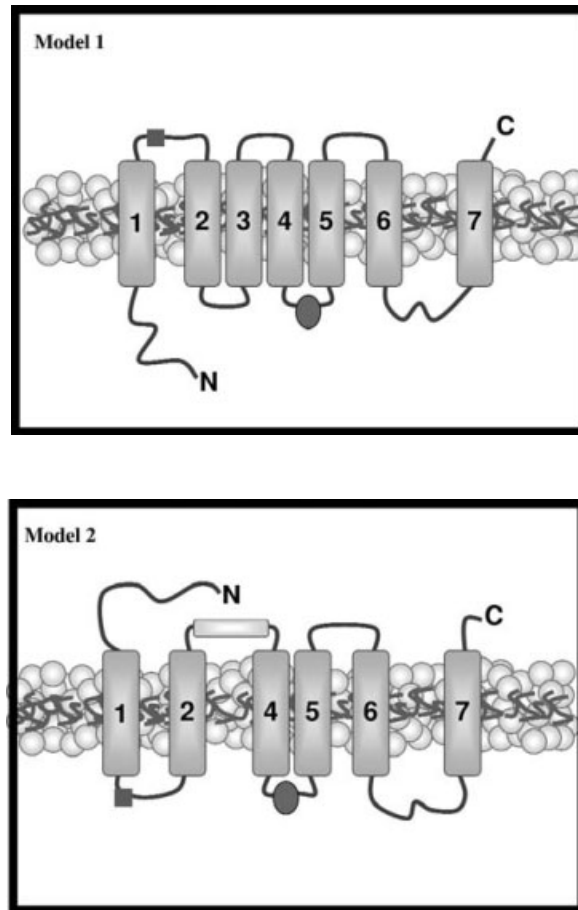


Fig. 10.5 Proposed models for the *XvSap1* protein. Blue boxes and blue lines indicate TMD and interconnecting loops, respectively. Red circle, phosphorylation site. Green box, position of the highly conserved DRY triplet motif. For color detail, please see color plate section.

with ABA and expression of *XvSap1* only within 24 hours of abiotic stress treatments. The qPCR also indicated that after 6 hours of *XvSap1* upregulation, there was a decrease in *XvSap1* expression between 12 and 24 hours followed by increased expression at 48 hours. Furthermore, a number of *X. viscosa* genes involved in protective mechanisms to abiotic stress such as antioxidants were upregulated only after 48 hours in response to the same ABA treatment. This suggests that *XvSap1* is involved in the initial and late stages of the protective response to abiotic stress. In support of this, a putative ABA-responsive element (ABRE) has also been found in the promoter region of the *XvSap1* gene (data not shown). The ABRE has previously been shown

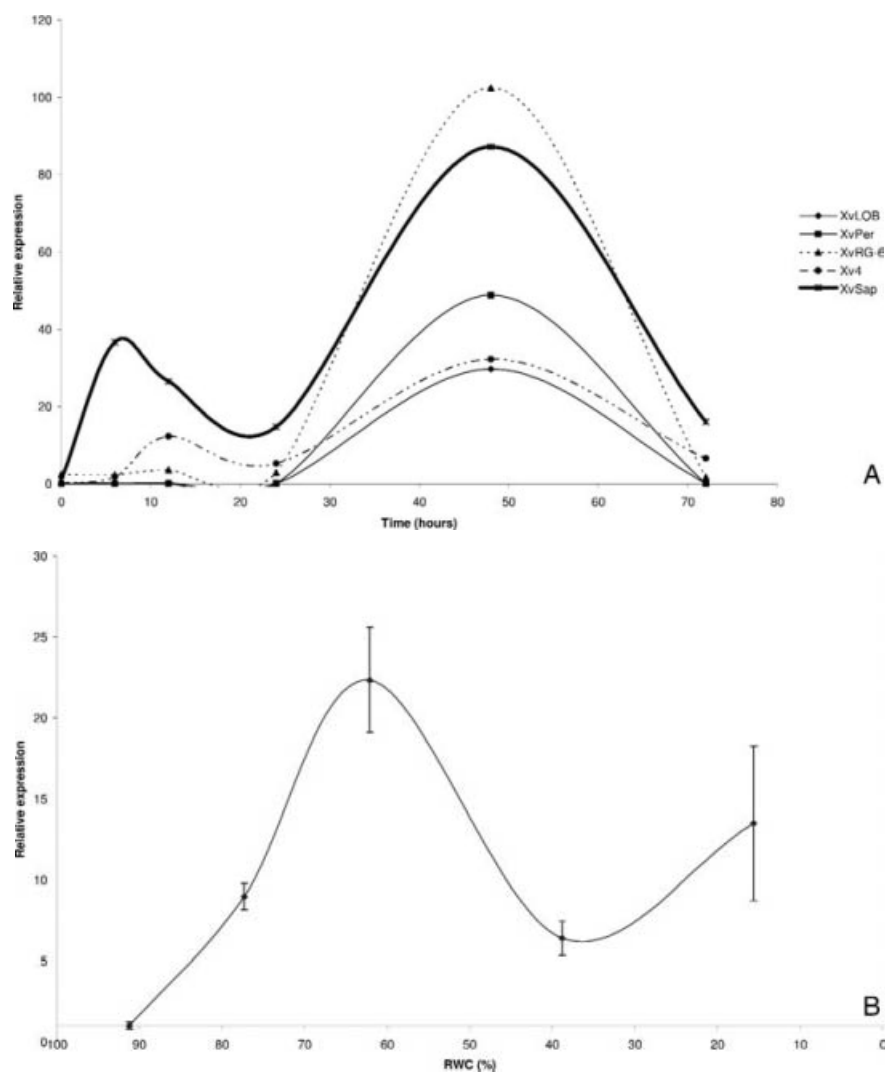


Fig. 10.6 Expression profile curves of *XvSap1* transcripts using qPCR analysis. (A) Expression profile following exogenous treatment of an *X. viscosa* plant with ABA. The *XvSap1* profile is displayed in *bold*. (B) Expression profile following a dehydration treatment of an *X. viscosa* plant. For color detail, please see color plate section.

to confer ABA responsiveness to certain genes (Marcotte et al. 1989). For the dehydration stress, qPCR analysis indicated that *XvSap1* mRNA was upregulated at 60% RWC. Thereafter, expression decreased but increased again at 15% RWC. This would suggest that *XvSap1* is involved in the initial and late stages of the protective response to dehydration.

Based on the bioinformatics analyses and expression studies, Breton et al. (2003) hypothesised two putative functions for the COR413 protein family. The first hypothesis suggests a role in stabilising the plasma membrane lipid bilayer, while the second and more intriguing hypothesis suggests that COR413 proteins are involved in environmental stress signaling. The second hypothesis was based on comparing the structural model of COR413 proteins to that of the mammalian rhodopsin G protein-coupled receptor (GPCR) family.

The GPCRs are a protein family of transmembrane receptors that transduce an extracellular signal into an intracellular signal. The GPCRs are integral membrane proteins that possess several membrane-spanning domains or transmembrane helices. The GPCRs are the largest protein family known with a diversity of functions due to the wide range of ligands recognised by members of the family, from photons to small molecules and proteins. The mechanism of signal transduction is poorly understood but is known that inactive heterotrimeric G proteins (α , β , and γ) are bound to the GPCR in its inactive state. The $G\alpha$ subunit contains a RAS-like domain that has a GDP/GTP nucleotide binding site and GTPase hydrolase activity. Once a specific ligand is recognised, the GPCR shifts conformation and mechanically activates the heterotrimeric $G\alpha$ subunit to bind GTP. The $G\alpha$ -GTP complex dissociates from the $G\beta/G\gamma$ subunit dimer with $G\alpha$ and/or $G\beta\gamma$ detaching from the GPCR. The heterotrimeric G proteins then transduce a signal by interacting with downstream effector molecules. Thus, the GPCR exists in a conformational equilibrium between active and inactive states. Signal pathways that can be activated by heterotrimeric G proteins range from activating ion channels, kinases, and phospholipases to increasing or inhibiting cAMP levels. Previously, it was thought that heterotrimeric G proteins are required for signal transduction; recently, however, there is evidence to suggest that some GPCRs are able to signal without G proteins (Kroeze et al. 2003). Furthermore, for certain metazoan GPCRs, dimerization of GPCR is also part of the activation process and receptor regulation (Gether and Kobilkas 1998).

Regulation of GPCR is done via kinases that phosphorylate sites, typically situated on the last intracellular loop, of the receptor. The GPCR kinases phosphorylate activated GPCRs, which in turn are bound by β -arrestin proteins that inhibit interaction with G proteins and cause receptor desensitization (Flanagan 2005). Protein kinases A and C also phosphorylate GPCRs and cause receptor desensitisation (Jones and Assman 2004). The G protein subunits can also be regulated via G protein signaling (RGS) proteins, which are GTPase activating proteins. Only a few components of the G protein signaling system have so far been identified in plants.

The triplet GPCR conformational motif Glu/Asp-Arg-Tyr (E/D-R-Y) at the cytoplasmic end of the third TMD is one of the few motifs that seem to be highly conserved among GPCRs and serve to constrain the inactive conformation (Parnot et al. 2002). There is evidence that the E/D-R-Y motif interacts

directly with the G proteins (Wess 1998). Mutagenesis studies have shown that the acidic side chain (Asp/Glu) regulates receptor activation and coupling of agonist binding to activation of G protein signaling (Flanagan 2005). Another important structural feature found in GPCRs is highly conserved proline residues located inside the TMD, which allow for correct folding and function (Deupi et al. 2004). These proline residues induce a significant distortion in the TMD helices referred to as the proline-kink allowing conformational rearrangement of the TMDs.

The GPCRs display little or no similarity between subfamilies (Pierce et al. 2002) and have been defined by functionality and not by sequences similarity (Perfus-Barbeoch et al. 2004). Both the *XvSap1* and COR413-PM proteins have the signature TMD profile (Figs. 10.2 and 10.4), an intracellular phosphorylation site, conserved proline residues, and a motif similar to the E/D-R-Y motif (Fig. 10.1). Furthermore, transcriptional analyses of *XvSap1* expression (Fig. 10.6) demonstrate clearly that *XvSap1* is associated with and regulated by environmental stress. Taken together, it raises the suggestion that *XvSap1* may be or act as a putative GPCR in *X. viscosa* for a specific ligand or ligands associated with osmotic stress.

In plants, G protein-coupled signaling has been extensively reviewed (Assmann 2005, Jones and Assmann 2004, Perfus-Barbeoch et al. 2004, Jones 2002). In *A. thaliana*, heterotrimeric G protein signaling has been linked to ABA responsiveness and an *A. thaliana* GPCR, GCR1, which was shown to interact with a $G\alpha$ protein (GPA1) and act as a negative regulator of GPA1-mediated ABA responses in guard cells (Pandey and Assmann 2004). The *A. thaliana gcr1* mutant plants exhibited hypersensitivity to ABA and were more drought tolerant than wild-type plants. No ligand of GCR1 has yet been identified, although the *gpa1* knockout mutant displays reduced sensitivity to a sphingolipid, sphingosine-1-phosphate (S1P).

Plant sphingolipids that are localized to the plasma membrane and tonoplast account for approximately 10% of the total lipid content, with the predominant plant sphingolipids being ceramides, glucosylceramides, glycerophosphosphingolipids, and inositol-phosphorylceramide (Dunn et al. 2004). Recent studies indicate important functions for sphingolipids and their metabolites in plant cell-cell interactions, membrane structure (Lynch and Dunn 2004), and novel plant-specific cell signaling pathways (Worrall et al. 2003). Coursol et al. (2005) demonstrated that the enzyme responsible for S1P production, sphingosine kinase (SphK), is triggered by ABA in the guard cells and is involved in both inhibiting the opening and promoting the closure of the stomata. The S1P-mediated stomatal closure during drought prevents water loss from leaves and decreases CO₂ uptake into leaves. Furthermore, they demonstrated that this occurred via GPA1 (heterotrimeric G protein) as the S1P stomatal closure was impaired in the guard cells of a *gpa1* knockout plant, although the ABA-stimulated SphK kinase activity was retained. The demonstration

that GCR1 interacts with GPA1 (Pandey and Assmann 2004) and that S1P is a high-affinity ligand for the mammalian S1P4 GPCR (Candelore et al. 2002) suggests that GCR1 is the receptor for the S1P ligand. If this were the case, the *gcr1* and *gpa1* mutant guard cell response to ABA and S1P would be the same. However, the *gcr1* guard cells exhibit hypersensitivity, whereas the *gpa1* guard cells exhibit insensitivity to these ligands (Assmann 2005). This suggests that although S1P could be a possible ligand for GCR1, GPCR signaling in plants can occur through G protein-independent pathways. For example, phospholipases such as phospholipase D (PLD) and phosphatidylinositol-phospholipase C (PLC) also act as intracellular effectors of G protein signaling via ABA. In addition in *A. thaliana*, PLD α 1 directly binds GPA1 via a motif similar to the E/D-R-Y motif present in GPCRs (Wang 2004). This binding inhibits PLD α 1 activity that is relieved upon GTP addition. During freezing, *A. thaliana* pld α 1 mutants displayed less phospholipid degradation and greater freezing tolerance than wild-type plants (Welti et al. 2002).

Sphingolipids have also been implicated in freezing and chilling tolerance in plants and in the GPI anchors (Dunn et al. 2004). A subset of GPI anchors in plants possesses sphingolipid anchors, and *A. thaliana* GPI anchored proteins have functions related to signaling, response to stress, and cell wall remodeling (Lynch and Dunn 2004). The GPI anchors are usually targeted to sphingolipid enriched membrane microdomains (rafts), which are involved in signal transduction (van Meer and Lisman 2002). Microdomains isolated from tobacco leaf plasma membrane were also found to contain the G protein β subunit (Peskan et al. 2000). As mentioned previously, although COR413-PM proteins possess GPI anchors, our analyses of *XvSap1* indicate that this protein lacks GPI anchors.

The data from Garwe et al. (2003) and our current research, although contradictory with respect to expression profiling of ABA, present three hypotheses. First, *XvSap1* may be involved in stabilizing the plasma membrane; second, it may be involved in maintaining ion homeostasis; and last, *XvSap1* may be a GPCR-like receptor associated with signal transduction in osmotic stress.

A structural role for *XvSap1* in membrane stability is a still a very strong hypothesis as *XvSap1* expression in *E. coli* (srl::Tn10) mutants conferred stress tolerance to osmotic stress (Garwe et al. 2003). As bacteria do not seem to possess GPCRs, the question may be raised as to how an osmotic stress signal would be transduced via *XvSap1* in *E. coli* in the event that *XvSap1* is a GPCR. As mentioned previously, there is evidence that GPCR signaling can occur through G protein-independent pathways. However, Marlovitis et al. (2002) also provided evidence that a transmembrane protein (FeoB) required for iron uptake in bacteria has a covalently linked G protein, which was able to bind and hydrolyse GTP. The authors also speculate that FeoB may be the primordial archetype of G protein-regulated membrane proteins. This suggests that G protein signaling could still operate in a prokaryote but still raises

questions as to what ligand would act on *XvSap1*. In prokaryotes, possible stimuli for sensing osmotic stress are turgor, membrane strain or shrinkage, external or internal osmolarity, ion concentration, or the transmembrane osmotic gradient (Heermann and Jung 2004). In *E. coli*, it has been suggested that these primary stimuli mechanically perturb osmoreceptors so that the signaling can be initiated (Vaknin and Berg 2006).

The research into GPCR/G protein signaling and osmotic stress perception in plants is relatively new, and there is still much research needed to understand what molecular components are involved and which networks underlie these signaling pathways. Our current work involves creating point mutations in *XvSap1* of the identified GPCR-associated motifs and residues and investigating the impact of these mutations on osmotic stress tolerance in *E. coli* and transgenic *A. thaliana*. We also intend to investigate the proteomic profile of the *E. coli* mutant expressing *XvSap1* in order to determine whether there is regulation of osmotic stress mechanisms or whether *XvSap1* primarily serves a structural role.

We have already demonstrated that *XvSap1* is regulated by environmental stress and that it is able to increase salt and desiccation stress tolerance when expressed in *A. thaliana*. By elucidating the mechanism, whether it be structural, signaling, or ion homeostasis, whereby *XvSap1* is able to protect the plant against osmotic stress, we will be able to refine our approach in developing transgenic crops expressing *XvSap1*.

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